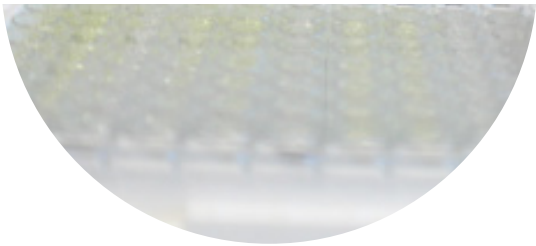
U-CyTech BV
Yalelaan 48
3584 CM Utrecht
The Netherlands
P +31.30.253.5960
F +31.30.253.9344
INFO @ucytech.com
www.ucytech.com

# Instruction Manual
# Human Autoantibody Assay

1-plate format



*For research use only.*
Not for use in diagnostic or therapeutic procedures.

# Contents



# Abbreviations

| | |
|---|---|
| AP | Alkaline Phosphatase |
| CV | Coefficient of Variation |
| EDTA | Ethylenediaminetetraacetic acid |
| h | hour |
| Ig | Immunoglobulin |
| IL | Interleukin |
| min | minutes |
| N | Negative control |
| NCCLS | National Committee for Clinical Laboratory Standards |
| OD | Optical Density |
| pNPP | p-NitroPhenyl Phosphate |
| RT | Room Temperature |
| S | Sample |
| Std | Standard |
| U | Unit(s) |

# Introduction

Naturally occurring autoantibodies are antibodies that bind and react with “self“ molecules originating from the body’s own cells, tissues or organs. Most autoantibodies are produced by the immune system when it fails to distinguish between “self” and “non-self.” This may cause inflammation, damage, and/or dysfunction of organs or systems, leading to signs and symptoms of autoimmune disorders. Autoantibodies occur across a range of infectious, hematologic, autoimmune, pulmonary and cancer diseases and some of them do not cause tissue injury directly but are thought to be part of an overall inflammatory response. Their presence in the blood can indicate that an autoimmune process is ongoing, for example antibodies relating to type 1 diabetes (T1D). These autoantibodies often appear years before clinical onset of T1D. There are also an increasing number of reports describing the presence of autoantibodies to a variety of cytokines in the circulation of human individuals without any obvious adverse health effects. Deregulation of this immune network, leading to enhanced autoantibody production has been described, resulting in a pathogenic effect. For example, the presence of high levels of neutralizing anti-IFN-γ autoantibodies can underlie disseminated non-tuberculous mycobacterial infections. The increased autoantibody production may be linked with a genetic predisposition combined with an environmental trigger, such as a viral infection or prolonged exposure to certain toxic compounds.

U-CyTech has developed several highly sensitive immuno-enzymatic capture assays for the quantitative determination of autoantibodies in human serum and plasma. The assay provides an attractive tool to screen or monitor disease progression and/or response to therapy. The performance of the assay is easy and straightforward and therefore simple to implement. The design of the supplied precoated plate is optimized to preserve the native conformation of the target analyte and avoids steric hindrance of antibody binding. In addition, the assay includes an internal negative control for each sample analysis, thereby minimizing the chance on false positivity. The offered autoantibody assays are further characterized by their high sensitivity, reproducibility and flexibility allowing the in depth study of human autoantibodies in health and disease.

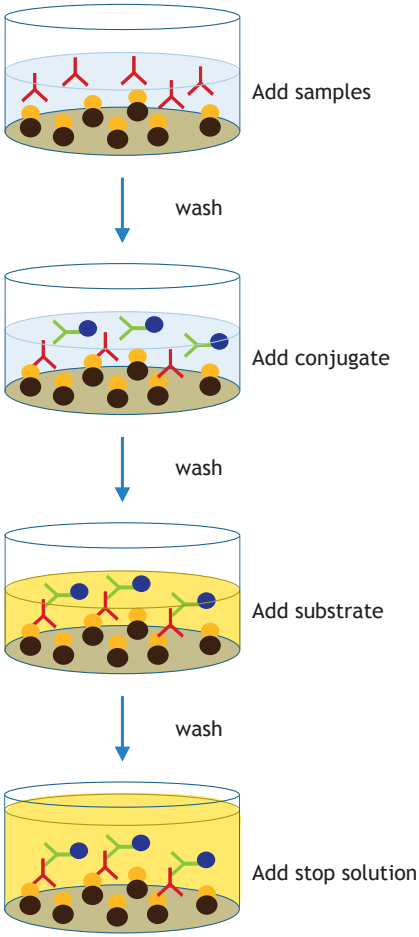
# This manual applies to the following Autoantibody Assays

| Analyte | Human |
|---|---|
| IFNγ | CT870 |
| Insulin | CT871 |

Overview of U-CyTech catalogue numbers of Autoantibody Assays.

# Principle of the test

U-CyTech Autoantibody kits are sensitive antigen capture assays for autoantibody determinations in human serum and plasma samples. The assay consists of streptavidin-coated 96-well strip plates with immobilized biotin-labeled molecules of the analyte of interest (antigen). Control wells contain an immobilized control agent for measuring non-specific binding. Standards, controls and samples are added to the wells, and (auto)antibodies present in the diluted samples bind to the captured antigen. Next, wells are washed and incubated with an alkaline phosphatase (AP)-labeled conjugate. After washing away unbound conjugate, the enzymatic activity is detected by addition of a ready-to-use p-NitroPhenyl Phosphate (pNPP) substrate. Finally, the enzymatic reaction is stopped and the optical density (OD) is read at 405 nm (reference 650 nm).



# Warnings and precautions

- This kit is designed for research use only, and not for diagnostic or therapeutic procedures.
- When blood components or other biological materials are used, then please note that all these materials should be considered as potentially infectious and handled with the usual precautions under Bio-Hazard conditions. Follow universal precautions as established by the US government agencies, Centers for Disease Control and Prevention and Occupational Safety and Health Administration, when handling and disposing of (potentially) infectious waste.
- Do not use reagents after the kit has exceeded the expiry date.

## Hazard information

All kit components are not classified as dangerous according to Regulation (EC) no. 1272/2008 and its amendments.
Please find the Material Safety Data Sheet on www.ucytech.com/manuals.

# Contents of the kit

| Items | Quantity (1-plate format) | Storage conditions |
|---|---|---|
| Precoated 96-well strip plate* (sensitized by analyte of interest and a control agent) | 1 plate | 4 ºC |
| Standard** | 1 vial | 4 ºC |
| Control High** | 1 vial | 4 ºC |
| Control Low** | 1 vial | 4 ºC |
| Sample dilution buffer (5x) | 1 vial (10 ml) | 4 ºC |
| AP conjugate (100x) | 1 vial (0.15 ml) | 4 ºC |
| Conjugate buffer (5x) | 1 vial (2.5 ml) | 4 ºC |
| pNPP substrate solution (ready-to-use) | 1 vial (14 ml) | 4 ºC in the dark |
| Stop solution (0.1 N NaOH) | 1 vial (14 ml) | 4 ºC |
| Wash buffer (20x) | 2 vials (30 ml) | 4 ºC |
| Adhesive cover slips | 5 pieces | RT |

* Consists of 12 separate strips of 8 wells fixed in a frame.
** Lyophilized.

# Storage and stability

**Precoated plate**
The precoated 96-well strip plate in the vacuum-closed sachet can be safely stored at 4 ºC until the expiry date (indicated on the label of the sachet). Do not use the plate when the sachet has lost vacuum. After opening return any unused strips to the provided self-seal plastic bag including desiccant and seal. Store at 4°C and use within 10 weeks.

**Standard**
The vial with lyophilized standard can be safely stored at 4 ºC until the expiry date (indicated on the vial). After reconstitution, the standard is stable for at least 10 weeks at 4 ºC when kept sterile. The standard can also be divided into small aliquots for single use. These aliquots should be stored at ≤-20 ºC (stable for at least two years).

**Control High and Control Low**
The vials with lyophilized controls can be safely stored at 4 ºC until the expiry date (indicated on the vials). After reconstitution, the vials with controls are stable for at least 10 weeks at 4 ºC when kept sterile. The controls can also be stored at ≤-20 ºC (stable for at least two years).

**Sample dilution buffer (5x), Conjugate buffer (5x) and Wash buffer (20x)**
The vials with Sample dilution buffer, Conjugate buffer and Wash buffer can be safely stored at 4 ºC until the expiry date (indicated on the vials). After opening, these solutions are stable for at least 6 months at 4 ºC when kept sterile.

**AP conjugate (100x)**
The vial with AP conjugate is stable until the expiry date (indicated on the vial) when stored at 4 ºC in the dark. After opening, the reagent is stable for at least 6 months at 4 ºC in the dark when kept sterile.

**pNPP substrate solution (ready-to-use)**
The ready-to-use pNPP substrate solution should be stored at 4 ºC in the dark and is stable until the expiry date (indicated on the vial). Avoid exposure to direct light (sunlight and UV sources) and heat.

**Stop solution (0.1 N NaOH)**
The ready-to-use Stop solution should be stored at 4 ºC and is stable until the expiry date (indicated on the vial).

# Materials and equipment (required but not provided)

- Sterile distilled water.
- Pipetting devices for the accurate delivery of volume required for the assay performance.
- Tubes and containers/plates to prepare the solutions.
- Dry incubator with thermostatic control up to 37 ºC.
- Plate washer: automated or manual (squirt bottle, manifold dispenser).
- Reading device for microtiter-plate (wavelength set to 405/650 nm).
- Vortex mixer.

# Preparation solutions and reagents

**Precoated plate**
Bring vacuum-closed precoated plate to RT prior to use. The standards/controls/samples can be added directly into the wells, without prior washing. Ensure that strip-wells are firmly fixed into the frame provided and label the control agent strips (see page 14). After opening return any unused strips to the provided self-seal plastic bag including desiccant, seal and store at 4°C (use within 10 weeks).

**Wash buffer (20x)**
Dilute Wash buffer 1/20 in distilled water and mix thoroughly. At least 360 ml diluted Wash buffer is required for one plate. Always use freshly prepared Wash buffer.

**Sample dilution buffer (5x)**
Before use, mix the solution gently. Dilute the buffer 1/5 in distilled water. Mix gently but thoroughly. Always use freshly prepared Sample dilution buffer.

**Standard**
Reconstitute the lyophilized Standard by adding 1 ml of diluted Sample dilution buffer (1x) into the vial. Mix the solution gently for approximately 15 seconds and allow it to stand for 30 min at RT. Avoid vigorous shaking. Thereafter, the reconstituted Standard is diluted as described in "Preparing the standards" on page 11.

**Control High**
The Control High contains high levels of anti-human antibodies directed to the analyte of interest. The concentration is specified on the *Certificate of Analysis*. The vial with lyophilized control should be reconstituted in 1 ml diluted Sample dilution buffer (1x). Mix gently for approximately 15 seconds and allow to stand for 30 min at RT. Thereafter, the reconstituted control can be added on the plate without further dilution.

**Control Low**
The Control Low contains low levels of anti-human antibodies directed to the analyte of interest. The concentration is specified on the *Certificate of Analysis*. The vial with lyophilized control should be reconstituted in 1 ml diluted Sample dilution buffer (1x). Mix gently for approximately 15 seconds and allow to stand for 30 min at RT. Thereafter, the reconstituted control can be added on the plate without further dilution.

**Conjugate buffer (5x)**
Before use, mix the solution gently. Dilute the Conjugate buffer 1/5 in distilled water. Mix thoroughly. This buffer is required for the preparation of the AP conjugate working solution.
For one plate: 2 ml Conjugate buffer (5x) is mixed with 8 ml distilled water.

**AP conjugate**
Mix gently 1 volume of AP conjugate and 99 volumes of diluted Conjugate buffer (1x). Do not use a Vortex mixer.
For one plate: 100 µl AP conjugate (100x) is gently but thoroughly mixed with 9.9 ml diluted Conjugate buffer (1x).

**pNPP substrate solution (ready-to-use)**
Bring pNPP substrate solution to RT prior to use (keep in the dark).

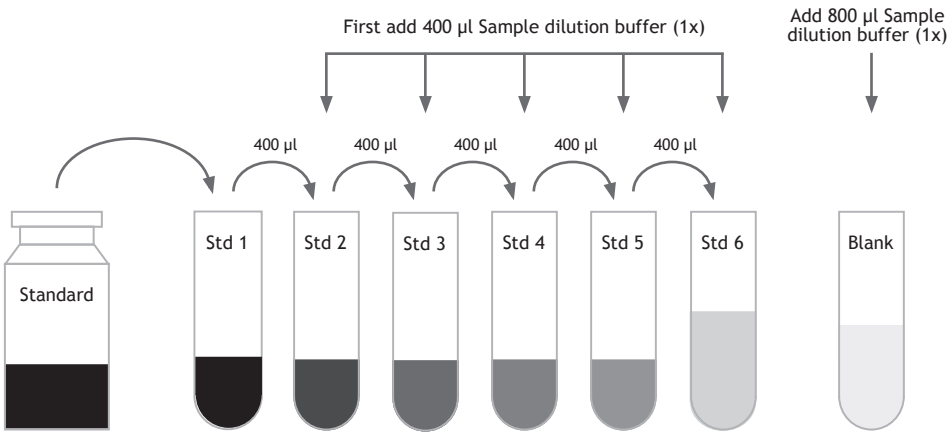
**Stop solution (ready-to-use)**
Bring Stop solution to RT prior to use.

# Preparing the standards

By making use of a standard curve, the autoantibody concentration can be determined in serum and plasma samples. The standard curve is generated from the data of 6 two-fold serial dilutions (Std 1-6) of the Standard. Always include a blank control (diluted Sample dilution buffer only) (See also page 14 for plate layout).

For one precoated Autoantibody plate:

- Take 7 tubes: Mark them Std 1 till Std 6 and blank. Add 400 µl Sample dilution buffer (1x) to 5 of these tubes (Std 2 till Std 6) and 800 µl to the last tube (Blank).
- Prepare in the remaining tube (Std 1) the highest concentration to be used in the standard curve (see *Certificate of Analysis*) by mixing an appropriate volume of Standard with Dilution buffer. The final volume of Std 1 should be 800 µl. Allow the mixture to stand for at least 15 seconds before adding it to the next tube.
- Perform serial two-fold dilutions by transferring 400 µl of Std 1 to the next tube (Std 2). Mix well and transfer 400 µl from Std 2 to the next tube Std 3, and so on until Std 6.




*Note: A standard curve, including a blank, should be run on each plate.*

# Specimen collection and handling

Specimens should be clear, non-hemolyzed and non-lipemic. Excessive hemolysis and the presence of large clots or microbial growth in the sample may interfere with the performance of the test.

- Serum: use a clot tube and allow sample to clot for 30-45 min at RT, then centrifuge for 10-15 min at 1,000-2,000 x g (RT) and collect serum immediately.
- Plasma: collect plasma by using anticoagulant, such as heparin (do not use EDTA). Mix well immediately after collection. Centrifuge for 10-15 min at 1,000-2,000 x g (RT) and collect plasma.

Samples should be aliquoted and stored frozen at ≤-20 ºC. If samples are run within 5 days, they can be stored at 4 ºC. Avoid repeated freeze-thaw cycles. Do not heat serum/plasma samples. Prior to analysis, frozen samples should be completely thawed and mixed well.

**Note:** *Specimen collection from humans should be carried out in accordance with NCCLS document M29-T2, "Protection of laboratory workers from infectious diseases transmitted by blood, body fluids and tissue".*

# Sample preparation

Both serum and plasma (do not use EDTA as anticoagulant) can be used. Make sure the samples are clear and homegenous (if not, filtrate or centrifuge the sample briefly for 5 min at 10,000 x g).

Use polystyrene tubes to prepare dilutions.

When the levels of autoantibodies present in the samples are unknown, it is recommended to prepare and analyze a series of dilutions to ensure that sample measurements fall within the assay range.

Dilute samples 100- to 500-fold in diluted Sample dilution buffer. Mix well and incubate for 30 min at RT.

The diluted samples are tested in triplicate as illustrated in the Plate lay-out as shown on page 14 (two wells are autoantibody-specific; one well is for the internal negative control).

# Directions for washing

- Incomplete washing of the wells will adversely affect the assay. All washing steps should be performed with diluted Wash buffer.
- Washing can be performed manually as follows: completely aspirate the liquid from all wells by gently lowering the tip of an aspiration device into each well (without touching the bottom). After aspiration, fill the wells with at least 250 µl Wash buffer and then aspirate the liquid. Repeat these steps at least six times. After washing, the plate is inverted and tapped dry on absorbent tissue paper.
  Alternatively, the Wash buffer may be put into a squirt bottle. If a squirt bottle is used, empty the wells by a firm 'shake-out' action and flood the plate with Wash buffer, completely filling all wells. Repeat these steps at least six times. After washing, the plate is inverted and tapped dry on absorbent tissue paper.
- When using an automated washing device, follow operating instructions carefully.

**Note:** *When you have too much airbubbles by using a squirt bottle, you can replace Wash buffer by PBS during the last washing step.*

# Data analysis

First, subtract the reference $OD_{650nm}$ from each $OD_{405nm}$ (=OD).

Calculate the mean OD for each standard concentration (n=2) (Std 1-6; see section “Preparing the standards” on page 10 and “Plate layout” on page 14). Next, calculate the mean $OD_{blank}$ (n=4) and subtract this value from the mean OD of each standard concentration (see formula below).

Formula: $OD = \text{mean } OD_{std\ 1\text{-}6} - \text{mean } OD_{blank}$

To create the standard curve plot the standard concentration (x-axis) versus the corresponding OD (y-axis). Draw the standard curve, using a 4-parameter logistic regression curve (for example, see *Certificate of Analysis*).

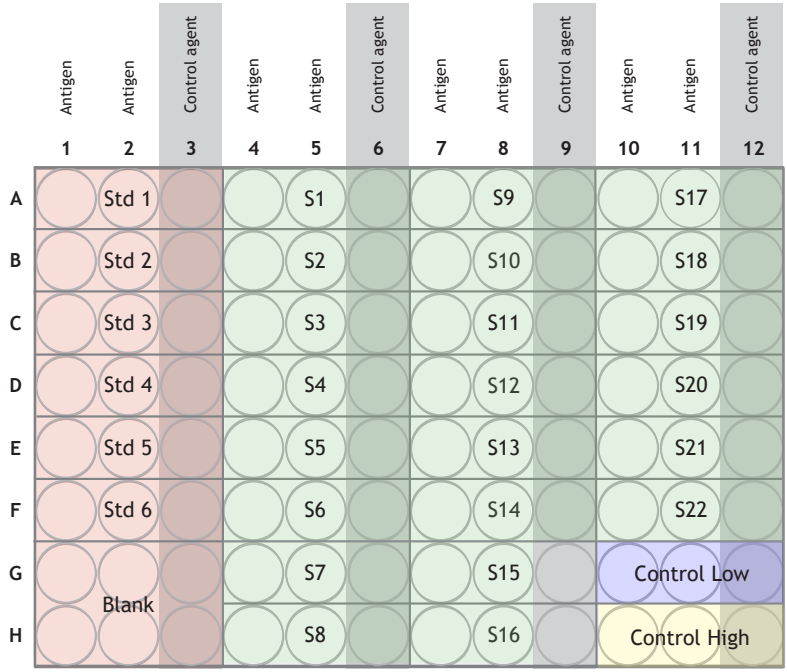
For samples, calculate the mean OD for each sample and substract the $OD_{control\ agent}$. The autoantibody concentration of samples can be determined from the standard curve by interpolation. The calculated concentration must be multiplied by the dilution factor.

Test results are valid if the OD for the highest standard, blank and calculated values of the Control High and Control Low comply with the reference values indicated under Specification on the *Certificate of Analysis* enclosed in each Autoantibody kit. If these quality control criteria are not met, the assay run is invalid and should be repeated.

**Note:** *The OD values of the standards in the column with control agent (page 14; column 3) are not used for calculations. The OD values should be <0.200.*

# Plate layout

The columns 1, 2, 4, 5, 7, 8, 10, 11 of the precoated plate are sensitized with the analyte of interest (Antigen). Columns 3, 6, 9 and 12 are sensitized with a Control agent ( ).
The control agent serves as a negative internal control (visualizing false positive signals).




Use the first three columns ( ) for Std 1 till 6 and the Blank. Samples S1 till S22 ( ) are pipetted in three neighboring columns (triplicates). For example; sample S1 in positions A4, A5 and A6. This results in two specific determinations for antibody binding to the analyte of interest and one non-specific determination as an internal negative control. Include on each plate one Control Low ( ) and one Control High ( ).

# Assay procedure

*Vacuum-packed plate and all solutions should be at RT prior to use.*

1. Take the precoated strip plate out of the vacuum-packed sachet and label the control agent strips. Check “Plate layout” on page 14.
2. Add 100 µl of diluted standards/samples and undiluted controls to each well, without prior washing.
3. Seal the plate and incubate for 2 hours at 37 °C.
4. Discard the content of the wells and wash the wells six times with Wash buffer (1x).
5. Add 100 µl of diluted AP conjugate to each well.
6. Seal the plate and incubate for 1 hour at 37 °C.
7. Discard the content of the wells and wash the wells six times with Wash buffer (1x).
8. Add 100 µl of pNPP substrate to each well and seal the plate.
9. Leave the plate for 20 min at 37 °C in the dark.

**Note:** *The substrate produces a soluble yellow end product.*

10. After substrate incubation, do not empty the wells. Stop the reaction by adding 100 µl of Stop solution.
11. Read the plate at 405 nm and for reference at 650 nm within 15 min after stopping the reaction.

*Assay time: Maximal 4 h*

**Note:** *If you only use a part of the plate, put the remaining unused strips in the accompanying plastic bag with desiccant and store at 4°C. Use the unused strips within 10 weeks after opening of the vacuum closed sachet.*

# Troubleshooting

| Problem | Possible cause | Solution |
|---|---|---|
| Poor consistency of replicates | Inaccurate pipetting | - Ensure accurate pipetting of volume and avoid air bubbles.<br>- Check pipettes. |
| | Inadequate mixing of reagents | - Mix reagents adequately. |
| | Inadequate washing | - Increase the stringency of washes (particularly after the AP conjugate incubation step). |
| | Too much airbubbles after using a squirt bottle for washing | - You can add a PBS soak step after 5 washing steps with Wash buffer. Use commercially available liquid PBS (pH 7.4). |
| | Evaporation of solutions | - Ensure precise sealing of the plate. |
| | Non-homogenous samples or with high particulate matter | - Mix samples thoroughly and remove particulates by centrifugation. |
| $OD_{blank}$ values higher than 0.2 | Incubation time of pNPP substrate solution is too long | - Incubation time of substrate should be 20 min. |
| | Improper storage of pNPP | - Store pNPP at 4 °C and protected from light (unreacted pNPP substrate appears colorless to pale yellow). |
| | Working solutions were contaminated | - Solutions should be clear and colorless. Use a clean container before addition into wells. |
| | AP conjugate dilution was too concentrated or left too long on the plate | - Ensure proper dilution of AP conjugate and incubation time. |
| No signal or low OD values for standards | Improper storage of AP conjugate | - Avoid prolonged exposure to light and heat.<br>- Store conjugate always at 4 °C. |
| | Incorrect incubation times or temperature | - Ensure proper incubation times.<br>- Reagent solutions should be at RT before use. |
| | Improper quality or pH of distilled water | - Use distilled water, and not tap water.<br>- Check quality and pH of distilled water. |
| | Improper standard dilution | - Ensure proper dilution of standard. |
| | Degradation of antibodies | - Follow recommended storage conditions. |
| | Overly high washing / aspiration pressure from automated plate washer. | - Check function of washing system or apply manual washing. |
| Poor standard curve (linearity and dynamic range) | Improper standard dilutions | - Ensure proper dilution of standards (follow 'two-fold dilutions' guidelines). |
| | Inaccurate pipetting | - Ensure accurate pipetting of volume and avoid air bubbles.<br>- Check pipettes. |

# Notes

# Notes

# Notes

# Technical assistance

If you require assistance, information or have any questions, please contact our company:

**U-CyTech biosciences**
Phone: +31.30.253 5960
E-mail: info@ucytech.com