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# ECDTL2
# Test kit instructions for use

Version 13
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EarlyCDT® LUNG

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## 1. Intended use

The EarlyCDT Lung test kit is intended to be used as an immunoassay for the *in vitro* detection of a panel of seven lung cancer autoantibodies in humans to aid in the early detection of lung cancer in high-risk patients and risk stratification of patients with indeterminate pulmonary nodules identified by CT.

The EarlyCDT Lung test kit is designed for professional use and analytical results should be interpreted by medical professionals in combination with all other available clinical information. The test is intended for use with human serum collected in either clot activator or gel tube or plasma collected in EDTA tubes.

## 2. Introduction and background to the test

Lung cancer is a leading cause of all cancer related deaths worldwide affecting millions of patients annually. Lung cancer is responsible for 13% of cancer cases and 19% of all cancer deaths in both genders worldwide.[1] Tobacco smoking is the predominant cause of lung cancer; however the following are also risk factors:

- Age
- Gender
- Smoking history
- Emphysema/COPD
- Family history of lung cancer in first degree relatives
- Environmental exposure including dust, asbestos and ionising radiation including radon gas

Data from the National Lung Screening Trial[2] performed in the USA has shown that earlier detection through annual screening with low dose computed tomography (LDCT) results in a 20% improvement in lung cancer mortality. Hence earlier detection saves lives. However, LDCT screening has disadvantages in that 96.4% of positive LDCTs are benign findings (false positives).[2] It also delivers a potentially harmful dose of radiation.

Tumour cells express proteins in altered or up-regulated forms compared with their normal (non-malignant) counterparts. These are known as tumour-associated antigens (TAAs) and some are shed into the circulation of the cancer patient. A number of researchers have proven that the cancer patient's immune system often recognises the altered state (e.g. mutation, over expression or aberrant glycosylation) of TAAs as non-self and mounts an antibody response against them. Thus this antibody response acts as an early amplified *in vivo* signal for the presence of TAA (and hence tumours) in the body. Such antibodies are known as autoantibodies (AAb) since they are raised to the host's own altered proteins and it is these that the EarlyCDT Lung test is designed to measure. Hence the results of the EarlyCDT Lung test can be used as an aid for early identification of the presence of lung cancer. In validation studies, the EarlyCDT Lung test has demonstrated high specificity (>90%)[3, 4] and is non-invasive requiring only the drawing of a blood specimen.

The assay is conducted using a number of manual and automated steps in conjunction with laboratory equipment commonly used in the execution of enzyme-linked immunosorbent assay (ELISA) tests. Diluted patient specimen is loaded into wells of the antigen coated assay plate and incubated. Following a series of reagent addition, incubation and washing steps, bound autoantibodies are finally detected by the addition of a colourimetric reagent, and the resulting optical density (OD) signal is measured using a spectrophotometric plate reader. The plate reader exports the output OD data to an Excel file for test result calculation. A USB device containing a locked Excel template is included in the kit. The results are manually transferred to the Excel template in which the test result is generated automatically. The assay applies cut-point thresholds to quantitative results to obtain an ordinal answer (Negative, Moderate, High), and as such is semi-quantitative.

## 3. Recommended patient groups

The EarlyCDT Lung test kit is recommended for use in humans who are at high risk of lung cancer due to a combination of age, gender, smoking history and other risk factors such as environmental exposures (dust, asbestos, radioactive substances), those with a history of emphysema/COPD, or family history of lung cancer in a first degree relative.

Freenome's current recommendations are:

- Patients that are ≥50 years of age with at least a 20-pack year smoking history (equivalent to smoking one pack of cigarettes per day for 20 years).
- Patients that are 40-49 years of age with at least a 20-pack year history plus at least one additional risk factor (see introductory section).

The EarlyCDT Lung test kit can also be used in conjunction with diagnostic imaging techniques to further assess the risk of lung cancer being present where indeterminate lung nodules have been detected but have not been diagnosed as malignant.

## 4. Limitations of use

Patients with a previous history of cancer of any type should not take the EarlyCDT Lung test. The exception to this recommendation is for patients with a history of basal cell carcinoma (BCC). A study was conducted, and the data demonstrated that BCC does not impact the EarlyCDT Lung test result (data on file with Freenome).

The EarlyCDT Lung test kit should not be used in patients known to have diseases that result in an elevated level of serum total protein, for example myeloma, amyloidosis, monoclonal gammopathy of undetermined significance (MGUS).

## 5. Test principle

Patients with lung cancer can mount a humoral response to their disease[5-8] and autoantibodies have been described up to four years before clinical diagnosis in some cases[9-11]. The Freenome EarlyCDT Lung test kit is for the *in vitro* detection of autoantibodies to a panel of seven lung cancer antigens (CAGE, GBU4-5, HuD, MAGE A4, NY-ESO-1, p53 and SOX2) that are present from the earliest stages of lung cancer[3,12]. From pre-diagnostic lung cancer patient samples taken from the UKCTOCS clinical trial[13], it was shown that levels of the autoantibodies detected by EarlyCDT Lung could be elevated up to 9 years before clinical diagnosis of lung cancer with a median detection lead time of 4 years.[14]

The EarlyCDT Lung test kit is performed as an indirect Enzyme-Linked Immunosorbent Assay (ELISA). The reagents provided are used together for the measurement of the panel of the seven autoantibodies described above in up to ten patient specimens. Plates are pre-coated with tumour associated antigens and a control protein (VOL control) at two dilutions: 50nM and 160nM, see figure 1. Diluted patient specimen is loaded into wells of the coated plate and incubated. Up to five patient specimens can be run on each plate supplied. Following a series of reagent addition, incubation and washing steps, the autoantibodies are finally detected by the addition of a colourimetric reagent, and the resulting signal is measured using a spectrophotometric plate reader.

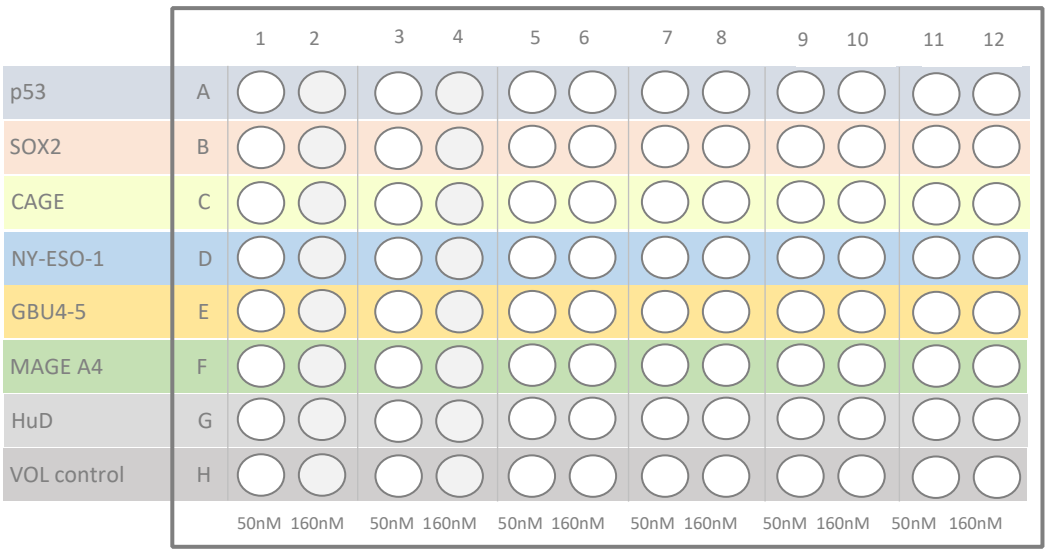


Figure 1: EarlyCDT Lung microtitre plate layout. One entire row is coated with the same TAA, alternating between 50nM and 160nM protein in each well. The wells of row H are coated with a control protein to allow for correction of non-specific binding.

## 6. Reagents and materials provided

- Each EarlyCDT Lung test kit contains the reagents listed in table 1, which are sufficient for a maximum of ten or eight tests depending on usage:
    - If both plates within a kit are being run at the same time, then up to ten patient specimens can be run in total:
        - Five patient specimens on plate 1 alongside Control A and five patient specimens on plate 2 alongside Control B
    - If plates within the kit are being run on different occasions, then up to eight patient specimens can be run in total:
        - Four patient specimens on plate 1, alongside Control A and Control B
        - Four patient specimens on plate 2, alongside Control A and Control B
    - Since partly used microtitre plates must be disposed of the most efficient use of this kit is achieved by running four or ten patient specimens at the same time.
- The expiry date of the kit is stated on the label outside the box.
- DO NOT use the kit beyond the expiry date.
- DO NOT use the kit if the outer seal is broken upon receipt and please contact earlycdt@freenome.com to report the occurrence.
- DO NOT use any opened or unopened reagents beyond their expiry date.
- DO NOT mix reagents from different kit lots.

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| Table 1. ECDTL2 EarlyCDT Lung test kit components | | | |
|---|---|---|---|
| **Part number** | **Component** | **Quantity** | **Storage and stability after first use** |
| ECDTL2-SD | Specimen diluent | 1x 27.5mL | +2 to +8°C for 4 weeks |
| | A phosphate buffered saline solution containing 0.1% bovine casein and 0.05% ProClin 950. | | |
| ECDTL2-SA | Secondary antibody | 1x 0.27mL | +2 to +8°C for 4 weeks (refers to undiluted stock) |
| | Purified immunoglobulin fraction of rabbit antiserum to human IgG conjugated with horseradish peroxidase. Provided as a 100x stock solution (approx. 0.02g/L) in a phosphate buffered saline solution with 0.1% bovine casein. Contains 0.05% ProClin 950. | | |
| ECDTL2-AD | Antibody diluent | 1x 27.5mL | +2 to +8°C for 4 weeks |
| | A phosphate buffered saline solution containing 0.1% bovine casein and 0.05% ProClin 950. | | |
| ECDTL2-WB | Wash buffer (20x) | 1x 55mL | +2 to +8°C for 4 weeks (refers to undiluted stock). Dilute before use. |
| | Concentrated solution of phosphate buffered saline and 2% Tween 20. Contains 0.05% ProClin 950. | | |
| ECDTL2-MTP | 96-well microtitre plates | 2x plates | Discard after first use |
| | Polystyrene 96-well plates coated with recombinant human proteins and bovine casein. Supplied dry in a foil pouch with desiccant, stabilizer and preservative. | | |
| ECDTL2-CA | Control A | 2x 1.8mL | Discard after first use |
| | A pool of human fluids diluted in specimen diluent to working concentration. Formulated to be reactive to p53, SOX2, CAGE and NY-ESO-1 antigens. The relative concentration of reactive immunoglobulins for each antigen (expressed in relative units) is provided in the Lot Specific Insert. Contains 0.1% ProClin 950. Requires no further dilution. | | |
| ECDTL2-CB | Control B | 2x 1.8mL | Discard after first use |
| | A pool of human fluids diluted in specimen diluent to working concentration. Formulated to be reactive to MAGE A4, GBU4-5 and HuD antigens. The relative concentration of reactive immunoglobulin for each antigen (expressed in relative units) is provided in the Lot Specific Insert. Contains 0.1% ProClin 950. Requires no further dilution. | | |
| ECDTL2-SEAL | Sealing strips | 4x strips | Discard after first use |
| ECDTL2-SUB | Substrate | 1x 27.5mL | +2 to +8°C for 4 weeks<br>Keep out of direct sunlight |
| | Substrate for horseradish peroxidase. Contains a ready-to-use preparation of TMB (3,3',5,5'-Tetramethylbenzidine). Requires no further dilution. | | |
| ECDTL2-SS | Stop solution | 1x 27.5mL | +2 to +8°C for 4 weeks |
| | Sodium fluoride solution at 1g/L. Provided ready-to-use. | | |

| | | | |
|---|---|---|---|
| ECDTL2-USB | USB device | 1x | +2 to +8°C or room temperature |
| | Contains: Instructions for Use (ECDTL2-IFU); Lot Specific Insert (ECDT2-LSI); Material Safety Data sheet; Certificate of Analysis; and EarlyCDT Lung test result calculation software. | | |

## 7. Equipment, consumables and materials required but not provided

To carry out the EarlyCDT Lung test, additional equipment, consumables and materials are required, which are not provided with the kit. These are listed in table 2.

**Table 2. Equipment, consumables and materials required but not provided**

| Item | Description |
|---|---|
| 1. Graduated measuring cylinder | 50mL & 1000mL: For measuring and preparing wash buffer and secondary antibody solution. |
| 2. Deionised water | For preparation of wash buffer. |
| 3. 50mL tube with cap | For use during secondary antibody dilution. |
| 4. Pipettes | 20µL: For patient specimen preparation.<br>100µL 8-channel pipette: For dispensing secondary antibody, substrate and stop solution into the wells in each column of the plate.<br>**Note: Freenome recommend the use of an electronic 50 – 1200µL multichannel pipette if available for dispensing reagents**.<br>100-1000µL: For secondary antibody preparation and patient specimen dispensing.<br>1-5mL: For patient specimen and secondary antibody dilution.<br>**Note: All pipettes used must be maintained and calibrated so that accuracy of dispensing can be assured.** |
| 5. Pipette tips | Suitable for accurate dispensing with the above-mentioned pipettes. |
| 6. Reagent troughs | 3 clean troughs required, up to 25mL capacity, for holding secondary antibody, substrate or TMB solution whilst pipetting with a multi-channel pipette. |
| 7. Microtitre plate shaker | With a shaking orbit of 1.5 to 4.5mm. Please refer to table 3 for the appropriate speed to use depending on shaking orbit. |
| 8. Microtitre plate washer or wash bottle | A dispensing wash bottle capable of holding 0.5L of solution.<br><br>Or, an automatic plate washer capable of performing 4 consecutive wash cycles with a fill volume of 300µL/well. |
| 9. Timer | For ensuring incubation steps are performed to the time specified in the assay protocol. |
| 10. Absorbent paper | To remove any residual liquid from the wells following washing. |
| 11. Microtitre plate reader | With a reading wavelength of 650nm and minimum operating range of 0-3OD. |

| | |
|---|---|
| | **Note: The test was validated on Tecan Infinite M200 plate reader** |
| 12. PC with USB connection and Microsoft Excel 2007 or newer | For test result calculation using the USB device supplied with the kit. |

**Note: If any automation of equipment is implemented by the laboratory running the EarlyCDT Lung test kit, this automation must be validated in accordance with laboratory quality management system procedures.**

**Table 3. Shaking speed to use based on shaking orbit**

| Shaking orbit (mm) | Speed (RPM) |
|---|---|
| 1.5-2.0 | 600 |
| 2.5-3.0 | 500 |
| 3.5-4.5 | 400 |

## 8. Stability and storage

The EarlyCDT Lung test kit is stable until the expiry date stated on the box label when reagents are stored as indicated at +2 to +8ºC.

Unopened kit reagents should be stored as instructed on the individual reagent packaging and are stable as supplied until the kit expiration date. Once opened, each reagent is stable for the duration stated in table 1.

Kits or kit components that have not been stored in accordance with the above conditions should not be used.

## 9. Indications of instability

The substrate should be colourless. A blue colour indicates that the reagent may have been contaminated and should be discarded.

## 10. Safety precautions

For *in vitro* diagnostic use:

- Blood products are potentially infectious and should be handled, stored, and disposed of according to local biohazard regulations.
- Control reagents supplied with this kit are of human origin. They have been tested and found to be non-reactive for HIV-1/2 Antibody, HCV Antibody and Hepatitis B Surface Antigen (HBsAg). Since no method can completely rule out the presence of blood borne pathogens, the handling and disposal of the control reagents from this kit should be performed as if they were potentially infectious.
- The material safety data sheet (MSDS) provides detailed information relating to the correct disposal, handling and hazards that are associated with the EarlyCDT Lung test kit. The MSDS is provided on the USB device supplied with the kit and is also available from Freenome (phone: +44 (0)115 784 0501 or email: earlycdt@freenome.com).

The following components: Secondary antibody, Wash buffer, Specimen and antibody diluent, Control A, Control B and 96-well microtiter plates are classed as skin sensitiser

(category 1A) and may cause an allergic skin reaction. Appropriate Personal Protective Equipment (PPE) must be used when handling these components. For more information, please review the provided Safety Data Sheets.

## 11. Specimen collection handling and storage

The EarlyCDT Lung test kit is intended for use with human serum or plasma, collected in one of the following tubes:

- Serum: either clot activator or gel tubes.
- Plasma: collected using EDTA tubes.
- **Note:** Plasma collected in heparin and citrate tubes **should not be used**.

Collect blood by venepuncture or finger stick and process according to the collection tube manufacturer's instructions to separate serum or plasma from clotted and cellular material. Whole blood specimens should be stored at room temperature (+18 to +25°C) and be processed within four days. If specimens of serum or plasma are not to be assayed immediately, they can be stored at either of the options: +2 to +8°C for 14 days or at -25°C to -15°C or at -85 to -65°C for up to 12 months. Specimens must not undergo more than 5 freeze thaw cycles. Bring frozen specimens to room temperature and mix thoroughly by gentle inversion before analysis.

## 12. Assay protocol

Assay notes and precautions to read BEFORE starting an EarlyCDT Lung test kit assay

| | |
|---|---|
| ⚠ | Ensure that the assay is performed at +18 to +22°C and that all reagents are at this temperature before use. |
| ⚠ | Each timed incubation step must be performed exactly for the duration stated. As soon as one plate is filled a timer must be started. |
| ⚠ | All equipment used must be adequately maintained, calibrated and validated to ensure correct functioning. |
| ⚠ | Ensure reagents do not get mixed together by using clean troughs for each reagent. |
| ⚠ | Do not use the kit if the outer seal is broken upon receipt. |
| ⚠ | Do not use any kit reagents if they are damaged, appear to have leaked or are open. |
| ⚠ | Do not store reagents in packaging other than those in which they were received. |
| ⚠ | The EarlyCDT Lung test kit has been developed to be performed in a laboratory environment by a competent laboratory technician*. Ensure a |

thorough understanding of the protocol is gained before starting an assay.

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Dispose of solutions, especially those that contain biological material, according to local disposal regulations.

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Please check the contents of the kit and compare it with the listing included in table 1 to confirm that all required components are present.

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Patient information can be entered into software supplied with this device. Users must ensure that the computer hardware utlised for running the kit software has adequate security and access measures implemented to meet local requirements for patient data protection. This should include physical and electronic user access control. The kit software does not require IT network access to operate.

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**Reliability of assay results cannot be guaranteed if there are any deviations from the protocol provided in this document.**

Please contact Freenome customer services by phone +44 (0)115 784 0501 or email earlycdt@freenome.com for support, if the outer seal is broken upon receipt, the kit or components have been stored out of specified range, or the reagents are damaged or have leaked.

*A competent laboratory technician is defined as an individual with experience of running ELISAs and is trained and competent in carrying out general laboratory procedures, such as buffer preparation and use of pipettes, and is able to meticulously follow instructions.

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## Step 1: Reagent and specimen preparation

⚠ Read important notes below associated with this step.

a. Record the LOT number stated on the kit box label and your specimen ID numbers in table 4 below:

**Kit LOT number:** _____ (this will be needed when using the result calculation software)

**Table 4: Specimen IDs**

| Plate 1 | Specimen ID | Plate 2 | Specimen ID |
|---|---|---|---|
| Specimen 1 | | Specimen 6 | |
| Specimen 2 | | Specimen 7 | |
| Specimen 3 | | Specimen 8 | |
| Specimen 4 | | Specimen 9 | |
| Specimen 5 | | Specimen 10 | |

b. Remove all components from the kit box and leave to equilibrate to +18 to +22°C for **at least two hours before use**.
c. Depending on the number of plates being used in the assay, prepare the assay reagents and patient specimens as detailed in table 5.

**Table 5: Assay reagent preparation**

| Preparation | 1 plate assay (Max 4 samples) | 2 plate assay (Max 10 samples) |
|---|---|---|
| **Wash buffer** | Mix 25mL of Wash buffer (ECDTL2-WB) with 475mL of deionised water | Mix 50mL of Wash buffer (ECDTL2-WB) with 950mL of deionised water |
| **Secondary antibody** | Mix 0.12mL of concentrated antibody (ECDTL2-SA) with 12mL of antibody diluent (ECDTL2-AD) | Mix 0.24mL of concentrated antibody (ECDTL2-SA) with 24mL of antibody diluent (ECDTL2-AD) |
| **Patient specimen** | Mix 20µL of patient specimen with 2.2mL of specimen diluent (ECDTL2-SD) | |

d. Remove either one or both microtitre plates from their foil pouches (Part Number: ECDTL2-MTP), depending on the number of patient specimens.

### Step 1 - important notes:

- Due to the high concentration of salt in the wash buffer supplied you may observe crystallisation. If this happens, simply warm prior to use to redissolve the salts by either placing the bottle directly into a water bath at 37°C or holding the sealed bottle under warm running water until crystallisation is no longer visible.

- It is recommended to briefly centrifuge the vial of concentrated secondary antibody conjugate for 5 seconds using a microfuge, or to tap the vial on the benchtop to dislodge any contents that may have become trapped in the lid of the vial during transport.
- Dilute the concentrated secondary antibody conjugate in a 50mL tube and cap. Mix by inversion 5 times before use.
- Invert patient specimens and controls several times to mix prior to use.
- Ensure a clean pipette tip is used to handle each patient specimen or control.
- Store reagents and diluted specimens at room temperature on day of use, otherwise store at +2 to +8°C.
- Ensure plates are equilibrated to room temperature **prior** to opening the foil pouch.

## Step 2: Control and patient specimen incubation

a. Ensure that plates are orientated so that the A1 well is at the top, left hand corner.
b. Depending on the number of plates being used in your assay, add the kit control and patient specimen to plate 1 and/or 2, as follows:

**One plate assay**

i. Following Plate 1 layout in figure 2 below, dispense 100µL of Control A (RED cap, Part Number: ECDTL2-CA) into every well of column 1 and 2.
ii. Dispense 100µL of Control B (BLUE cap, Part Number: ECDTL2-CB) into every well of column 3 and 4.
iii. Dispense 100µL of diluted patient specimen 1 into every well of column 5 and 6. REPEAT for patient specimens 2, 3 and 4 as shown for Plate 1.
iv. As soon as the plate has been filled, start a timer for 90 minutes. Go to step c).

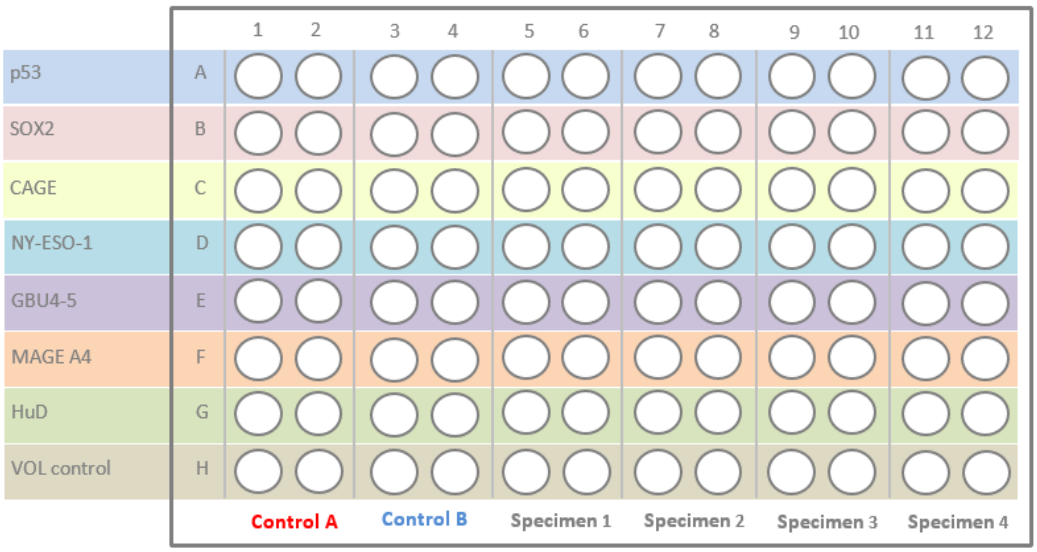


Figure 2: Plate layout when one microtitre plate is used in an assay.

## Two plate assay

i. Following Plate 1 layout in figure 3 below, dispense 100µL of Control A (RED cap, Part Number: ECDTL2-CA) into every well of column 1 and 2.
ii. Dispense 100µL of diluted patient specimen 1 into every well of column 3 and 4. REPEAT for patient specimens 2, 3, 4 and 5, as shown for Plate 1.
iii. As soon as Plate 1 has been filled, start a timer for 90 minutes.
iv. Following Plate 2 layout in figure 4 below, dispense 100µL of Control B (BLUE cap, Part Number: ECDTL2-CB) into every well of column 1 and 2.
v. Dispense 100µL of diluted patient specimen 6 into every well of column 3 and 4. REPEAT for patient specimens 7, 8, 9 and 10 as shown for Plate 2.
vi. As soon as Plate 2 has been filled, start another timer for 90 minutes. Go to step c).

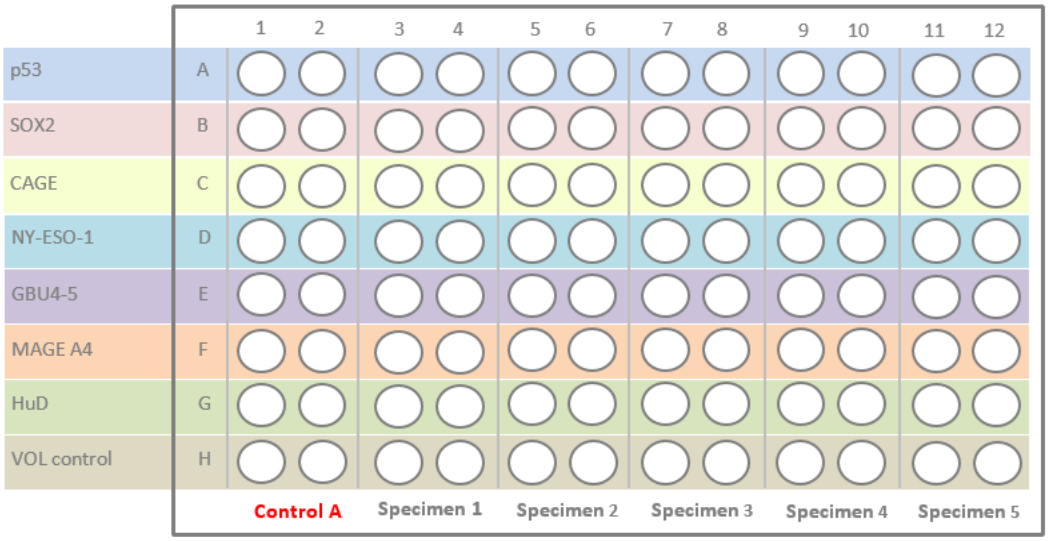


Figure 3: Plate 1 layout when both microtitre plates supplied with a single kit are used at the same time.

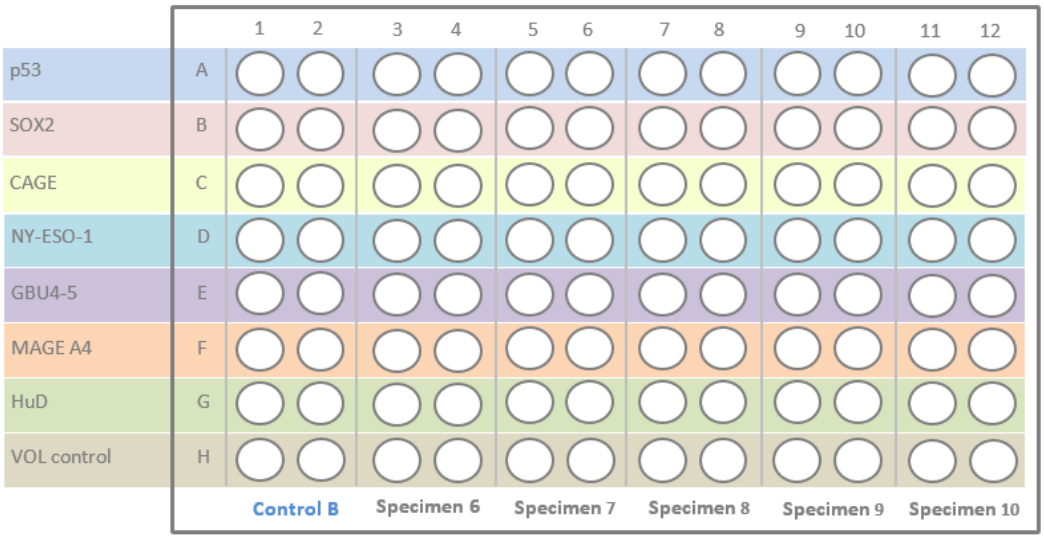


Figure 4: Plate 2 layout when both microtitre plates supplied with a single kit are used at the same time.

c. Once the control reagents and patient specimens have been added to the microtitre plate(s), cover each plate using a sealing film (Part Number: ECDTL2-SEAL), taking care to ensure the sealing film adequately covers all wells on the plate.
d. Place the covered plate on a microtitre plate shaker at the appropriate shaking speed (refer to table 3), incubate for a total of 90 minutes (as per timer already started) at +18 to +22°C.
    - If your plate shaker does not have a speed setting, use a 'moderate' shaking speed, this may need to be adjusted as necessary.

### Step 3: Secondary antibody addition

a. Carefully remove the sealing film from each plate and empty the well contents into a suitable biological waste container. Tap onto absorbent paper to remove all liquid.
b. Using a dispensing bottle, fill every well with prepared wash buffer and then empty into a suitable biological waste container. Firmly tap the plate onto absorbent paper to remove all liquid from the wells.
    - REPEAT this entire wash step a further three times.
    - If using an automated plate washer, carry out a four-cycle wash (300µL/well) using the wash buffer supplied with the kit.
c. Using an 8-channel pipette, dispense 100µL of secondary antibody working solution into every well of the plate, dispensing into each column sequentially (i.e., 1, 2, 3, 4…etc.).
d. As soon as one of the plates has been filled, start a timer for 60 minutes.
e. Cover each plate and place on a plate shaker. Incubate for 60 minutes (as per timer already started) at +18 to +22°C at the appropriate shaking speed.

### Step 4: Substrate addition

⚠ Read Important notes below associated with this step.

a. Carefully remove the sealing film from each plate and empty the well contents into a suitable biological waste container. Tap onto absorbent paper to remove all liquid.
b. Using a dispensing bottle, fill every well with prepared wash buffer and then empty into a suitable biological waste container. Firmly tap the plate onto absorbent paper to remove all liquid from the wells.
    - REPEAT this entire wash step a further three times.
    - If using an automated plate washer, carry out a four-cycle wash (300µL/well) using the wash buffer supplied with the kit.
c. Using an 8-channel pipette, dispense 100µL of substrate (Part Number: ECDTL2-SUB) into every well of the plate, dispensing into each column sequentially (i.e., 1, 2, 3, 4…etc.).
d. **Immediately** after the substrate has been dispensed into all 96 wells of the first plate, start a timer for 15 minutes and leave the plate to incubate at +18 to +22°C in the dark.
e. At the end of the 15-minute incubation, tap the side of the plate(s) for approximately 10 seconds to ensure the colour that has developed within each well is homogenous (i.e., no clumping is visible).

⚠ **Step 4 - important notes:**

- It is very important that the substrate is not left to incubate for longer than 15 minutes.
- Ensure exposure to light is limited as much as possible during the 15 minute incubation at +18 to +22°C.

## Step 5: Stop solution addition

a. Add 100µL of Stop Solution (Part Number: ECDTL2-SS) to every well, in the same order as the Substrate was added.
    - Note: there will be no visible colour change when the stop solution is added.
b. The microtitre plate can be read straight after the stop solution is added. To read the plate proceed to Step 6.
    - Note: The plates can be left for a maximum of 30 minutes at +18 to +22°C before reading.

## Step 6: Spectrophotometric measurement

⚠ Read important notes below associated with this step.

a. Insert the first microtitre plate into a plate reader, ensuring well A1 is placed in the top left corner.
b. Measure the optical density of each microtitre plate well spectrophotometrically at a wavelength of 650nm.
c. Export the outputted optical density (OD) values to an Excel file for test result calculation.

⚠ **Step 6 - important notes:**

- Do not read the plate using any wavelength other than 650nm.
- If a reference wavelength is required, we recommend using 480nm.

## Step 7: Test result calculation

⚠ Read important notes below associated with this step.

a. Insert the USB device (Part Number: ECDTL2-USB) into a PC and open the file ‘2 Plate Assay’ or ‘1 Plate Assay’ depending on how many plates were run.

b. Within the tab ‘Step 1. Data Input’, do the following:
    - Enter the kit LOT number into the field ‘Enter kit LOT’ (see figure 5(A)).
    - Enter the assay date into the field ‘Enter Assay Date’ (see figure 5(A)).
    - Enter an ID for each specimen in the appropriate field (see figure 5(B)).
    - Input the OD values from the Excel file generated in step 6 into the appropriate fields (see figure 5(C))

c. Within the tab ‘Step 2. Patient Information’, enter all relevant patient information.
d. Select the tab ‘Step 3a. Results’ to see the EarlyCDT Lung test result for each patient.
e. Select the tabs ‘Step 3b. Patient Report’ to print off a test report for each patient.

    - For information on what the test result means, see section ‘Interpretation of Results’, table 6, within this IFU, or refer to the information provided within the patient test report.

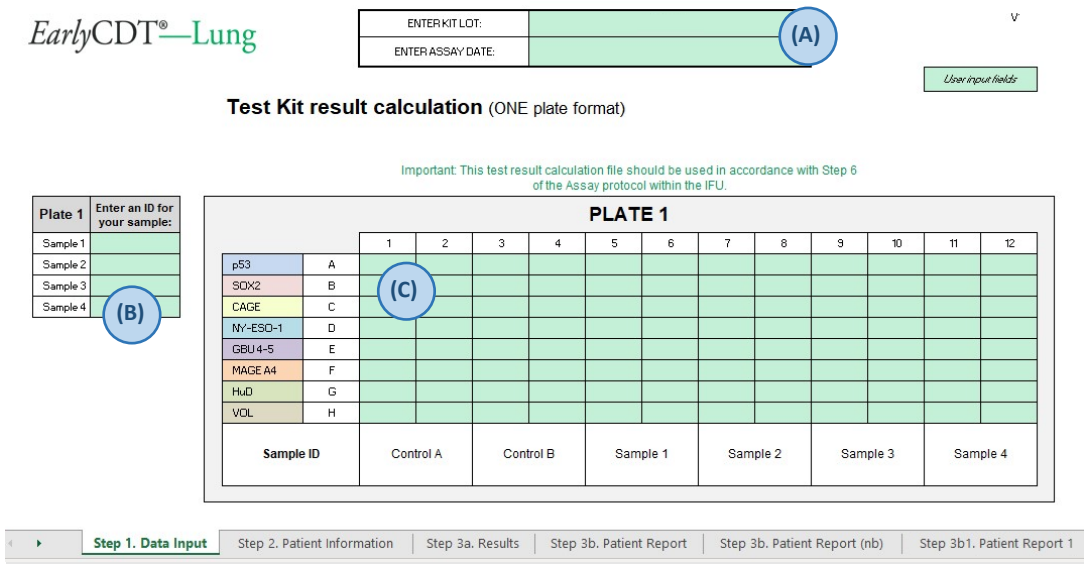


Figure 5. The opening page of the EarlyCDT Lung test kit result calculation software. (A) The correct kit LOT number must be entered in order to use the software. (B) Specimen IDs should be recorded. (C) Exported OD data is entered into the relevant fields.

### Step 7 - Important notes:

- The software loaded onto the USB device is specific to each LOT of EarlyCDT Lung test kits. Therefore, you must ensure that the USB device that came with the kit is used for result calculation. Failure to use the correct software will give an incorrect test result. The kit LOT number is stated on the kit's outer box label.

## 13. Calculation and Interpretation of results

The EarlyCDT Lung test kit requires no manual calculations as calculations are automated within the software provided on the USB stick within the kit.

The EarlyCDT Lung test kit is designed for professional use and analytical results should be interpreted by skilled medical professionals in combination with all other available clinical information. A moderate or high result indicates that the patient's risk of having lung cancer is greater than that predicted by their gender, age, smoking history and other risk factors alone. Estimates of the increased risk after a moderate or high positive test result are tabulated at the end of the patient report (the increase is larger after a high positive result than for a moderate positive result). The calculations were based on the addition of a diagnostic test to a baseline risk using the diagnostic likelihood ratio[15]. The increased risk may warrant a recommendation for additional testing, which could include CT imaging. A negative test result indicates that the patient's risk of having lung cancer is unchanged. Thus any estimates of lung cancer risk made prior to the test, based on risk factors alone, are still applicable. Table 5 provides a description of test results and terms that may be displayed within the kit software.

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**Table 6: Test result description of terms used within the kit software**

**QC acceptance range**

The controls used with the kit have an acceptance range which is defined within the 'Lot Specific Insert' included on the USB device supplied with the kit.

| Control Result | Description |
|---|---|
| **QC passed** | Control data falls within the acceptance range. |
| **QC failed** | Control data falls outside of the acceptance range and the test must be repeated. This may be due to a variety of reasons. Please contact Freenome for technical support on earlycdt@freenome.com |
| **Test Result** | **Description** |
| **High** | One or more autoantibodies in the EarlyCDT Lung panel are above the high cut-off value. The risk increase is very significant and is likely to influence the patient care pathway. |
| **Moderate** | One or more autoantibodies in the EarlyCDT Lung panel are above the low cut-off value but all are below the high cut-off value. The risk increase is significant and could be sufficient to influence the patient care pathway. |
| **No significant level of autoantibodies detected** | All autoantibodies in the EarlyCDT Lung panel are below the lower cut-off value.<br><br>**Note:** A test result of 'No significant level of autoantibodies detected' indicates a lower likelihood of lung cancer than a positive result, however it does not mean that the patient does not have, or will not develop, lung cancer. In order to be eligible for the test, the patient is already at an elevated risk of lung cancer as predicted by age, gender, smoking history and other risk factors. This result does not rule out the presence of lung cancer nor change the influence of these factors on the patient's risk. |
| **Invalid** | Unable to determine a result for this autoantibody. All other autoantibodies remain valid. If a plate or particular specimen has failed QC checks, then the test result for the respective patient specimen(s) will be "Invalid" and the EarlyCDT Lung Test should be repeated for those specimen(s). Please contact Freenome for technical support on earlycdt@freenome.com |

## 14. Summary of clinical study results

The EarlyCDT Lung test was clinically validated on four separate case control studies involving a total of 1153 lung cancer patients and 937 normal controls. Cases and controls were matched according to age, gender and smoking history. Clinical sensitivity and specificity of the two-stratum (negative, positive) seven autoantibody test was shown to be 35% and 91% respectively and the diagnostic likelihood ratios were also calculated, 3.81 for positive (DLRp) and 0.72 for negative (DLRn). In a screening context (1% prevalence) the accuracy was calculated to be 90%, the positive predictive value (PPV) to be 3.7% with a negative predictive value (NPV) of 99.3%. In a nodule context (25% malignancy rate) the accuracy was calculated to be 78%, the PPV 55.3% and the NPV 80.6%. The clinical performance characteristics were also calculated, assessing positivity as three strata (negative, moderate positive and high positive); the sensitivity and specificity were 14% and 94% for the moderate level positives and 21% and 97% for the high level positives. The respective DLRp were 2.1 and 8.7, DLRn 0.9 and 0.8. For a screening scenario (1% prevalence) the PPV was 2.0% for the moderate level and 8.0% for the high level positives. The respective NPV were 99.1% and 99.2% and accuracy 92.7% and 96.2%. In a nodule context (25% malignancy rate) the PPV was 40.6% for the moderate level and 70.9% for the high level positives. The respective NPV were 76.4% and 78.7%, and accuracy 73.5% and 78.0%.
These findings have since been confirmed in eight separate patient groups totalling 451 lung cancer patients and 8831 normal patients in real-world settings. The sensitivity and specificity of the device has also been demonstrated to be independent of stage or grade of lung cancer and was maintained for early-stage, late stage, small cell and non small cell lung cancer.[4]

In an audit of clinical outcomes of 1,613 US patients at high risk for lung cancer, whose physician ordered the EarlyCDT Lung, performance (sensitivity, specificity and overall accuracy) was consistent with the validation data. In the prevalence round >50% of cancers detected by a positive test were early-stage disease.[16]

A separate audit of the same patient cohort identified 269 patients with non-calcified pulmonary nodules identified by a radiologist within six months of taking the EarlyCDT Lung. Of this group, 52 patients were found to have lung cancer while the nodules identified in the other 217 patients were benign. Overall, a positive EarlyCDT Lung test was associated with a 2.2-fold increased risk of lung cancer and for patients with nodules in the 4 to 20mm size range (those that are more likely to be cured), relative risk of lung cancer was 2.7-fold. Additivity to the Gould, Brock and Swensen nodule-based risk models was also demonstrated for 4 to 20mm nodules.[17]

The EarlyCDT Lung test kit was clinically validated against the EarlyCDT Lung LDT in two case-control studies, the first with a total of 236 cancer cases (C) and 236 normal controls (N), split into training (154C/154N) and validation (82C/82N) phases, and a second larger study with a total of 366 cancer cases and 472 normal controls, also split into training (163C/163N) and validation (203C/309N) phases, to confirm similarity between LDT and kit, and to set diagnostic cut-offs for the kit. The EarlyCDT Lung kit performed in an equivalent manner to the EarlyCDT Lung LDT test with >90% concordance.

There is a significant shift to early-stage detection with EarlyCDT Lung testing. At two years, the ECLS clinical trial in Scotland demonstrated a significant reduction in late-stage detection in the EarlyCDT Lung intervention arm relative to the Control (standard of care) arm[16]. In the intervention arm, 33 out of 56 (58.9%) lung cancers were diagnosed at stage III/IV compared with 52 out of 71 (73.2%) in the control arm (37% reduction in late-stage). The hazard ratio for stage III/IV presentation was 0.64 (95% CI 0.41–0.99).[18]

## 15. Metrological Traceability

EarlyCDT is a functional assay giving values entirely specific to the particular ELISA measuring system. For the autoantibodies used in EarlyCDT no external reference materials with assigned potency are available. It is therefore not possible to assign absolute potencies to the assay calibrator materials or to the assay result itself. This means that it is not possible to prepare experimental materials of known concentration for studies of trueness, recovery or bias. In addition, no alternative functional assay of autoantibody titres is available for direct comparison with the EarlyCDT ELISA.

In place of external reference standards, a calibration system has been employed against human specimens which are known to contain antibodies corresponding to each analyte measured by the device.

Each EarlyCDT Lung test kit contains Quality Controls which are run by the user alongside patient sera every time the test is used. These controls (A & B) are pooled specimens of sera and pleural fluids which have been formulated to contain measurable levels of each analyte measured by the device and have an acceptance range which is determined during batch lot QC. The controls analyte levels must fall within that range for each run, otherwise the test result is invalid. If the controls are outside the acceptance range it signifies that the device performance is not to specification and must be repeated.

## 16. Mathematical Approach

Raw ODs are first corrected for non-specific binding by subtraction of the respective VOL result, and then calibrated to relative units (RUs) using pre-established antigen-specific standard curves. The standard curves were formed by plotting observed ODs versus a dilution series of calibrator fluids and then fitting four-parameter logistic models using non-linear regression. The conversion of observed ODs to predicted RUs uses the inverse formulae from the respective standard curve. A potency correction is applied to each new lot. Within each lot the standard curves remain fixed.

## 17. Reference Intervals

In the diseased state, autoantibody levels are expected to be elevated for at least one antigen in a proportion of the subjects. The decision as to the positivity of this elevation is not made on the basis of reference ranges but rather on optimized antigen-specific cut-offs. Hence, reference ranges or expected values in normal or affected populations are not presented, in order to avoid confusion as to the meaning of positivity.

## 18. Assay performance characteristics

### Accuracy and Trueness (Bias)

For analytes without available certified reference materials or reference measurement procedures, it is not possible to demonstrate accuracy or trueness. As there are no suitable external standards or reference materials for the assay these characteristics are not applicable and cannot be determined.

## Precision

Precision was determined using seven serum specimens known to contain positive autoantibody signals which were assayed six times on the same plate with each plate being run twice a day (separate kits) on six separate days. The repeatability or intra-assay precision of each autoantibody measurement was based on variation between replicates of the same sample on the same plate (and therefore on the same kit and day). Coefficients of variation (CV) for intra-assay precision, based on the variation between all replicates on the same day, and pooled over all plates over the six days, are shown in table 7.

The intermediate or inter-assay precision was based on variation between kits and plates run over several days on the same samples. CV for inter-assay precision, based on the variation between replicates on different days, are shown in table 8.

Precision for controls A & B are determined for every lot and provided with each kit.

**Table 7: Intra-assay precision**

| Autoantibody | 50nM | | 160nM | |
|---|---|---|---|---|
| | Mean OD | CV% | Mean OD | CV% |
| **p53** | 1.116 | 10.3 | 1.735 | 3.1 |
| **SOX2** | 2.443 | 3.1 | 2.782 | 2.4 |
| **CAGE** | 2.133 | 4.9 | 1.568 | 11.7 |
| **NY-ESO-1** | 3.046 | 1.4 | 3.039 | 1.3 |
| **GBU4-5** | 1.133 | 7.9 | 1.462 | 3.4 |
| **MAGE A4** | 1.418 | 4.0 | 1.723 | 2.1 |
| **HuD** | 1.352 | 12.9 | 2.073 | 2.4 |

**Table 8: Inter-assay precision**

| Autoantibody | 50nM | | 160nM | |
|---|---|---|---|---|
| | Mean OD | CV% | Mean OD | CV% |
| **p53** | 1.116 | 13.1 | 1.735 | 4.2 |
| **SOX2** | 2.443 | 3.8 | 2.782 | 3.1 |
| **CAGE** | 2.133 | 5.1 | 1.568 | 12.4 |
| **NY-ESO-1** | 3.046 | 3.3 | 3.039 | 3.6 |
| **GBU4-5** | 1.133 | 11.5 | 1.462 | 5.1 |
| **MAGE A4** | 1.418 | 5.6 | 1.723 | 3.3 |
| **HuD** | 1.352 | 12.9 | 2.073 | 4.5 |

**Measuring Range and Limits of Quantification**

The measuring range for the device's autoantibodies was determined to correspond to approximately 7.5% of the calibrator curve maximum for the lower limits of quantitation. The upper limits of quantitation were determined to be approximately 92.5% of the calibrator curve maximum or 3.0OD, whichever is lower, for all 7 autoantibodies measured by the device. It was also determined that the moderate and high cut-offs were within the quantitation limits for all of the antibodies which the device detects. However, calibration is batch specific (ref: section 16 Mathematical Approach above) and the exact limits of quantification and measuring range are also batch specific. Therefore, they are calculated for every lot and provided with each kit.

Test results outside this range are recorded as being below or above the limit of quantitation. Such values should still be assessed for the test result and are reported as 'No Significant Level of Autoantibodies Detected' or 'High' respectively.

**Analytical sensitivity, Limits of Detection and Limits of Blank**

Since independent reference materials of known autoantibody concentration are not available for this assay, it is not possible to assess analytical sensitivity.

The Limit of Detection (LoD) is defined as the lowest concentration of analyte that can be consistently detected, but not necessarily quantified, under routine clinical laboratory conditions in typical samples.

The Limit of Blank (LoB) is defined as the highest measurement result that is likely to be observed with a stated probability for blank samples.

The LoD and LoB for each autoantibody to each concentration of the corresponding antigen used in the test were determined and are shown in table 9. All values were substantially below the respective cut-offs, also shown.

**Table 9: Limits of Detection and Limits of Blank**

| Autoantibody | Antigen Concentration (nM) | LoB (RU) | LoD (RU) | Moderate cut-off (RU) | High cut-off (RU) |
|---|---|---|---|---|---|
| p53 | 50 | 0.88 | 1.51 | 3.58 | 4.652 |
| | 160 | 1.85 | 2.49 | 5.50 | 6.049 |
| SOX2 | 50 | 0.87 | 1.51 | 6.62 | 6.785 |
| | 160 | 2.21 | 2.88 | 7.82 | 8.146 |
| CAGE | 50 | 0.65 | 1.32 | 4.46 | 4.570 |
| | 160 | -0.54 | 0.13 | 3.73 | 4.181 |
| NY-ESO-1 | 50 | -1.25 | -0.61 | 3.01 | 3.158 |
| | 160 | -1.86 | -1.26 | 2.40 | 3.252 |
| GBU4-5 | 50 | 1.24 | 1.91 | 5.27 | 5.593 |
| | 160 | -0.03 | 0.60 | 2.82 | 4.357 |
| MAGE A4 | 160 | 1.22 | 2.05 | 4.94 | 5.101 |
| HuD | 160 | 4.42 | 4.96 | 8.43 | 9.703 |

**Linearity**

Specimens known to contain high levels of autoantibodies specific for one or more of the EarlyCDT Lung panel antigens were serially diluted in assay buffer and assayed using the EarlyCDT Lung protocol described above. Assuming the lowest dilution of the specimens to be 100%, predicted specimen dilution was plotted against known dilution. Slope and correlation coefficients ($R^2$) for representative signal specimen are given in table 10. All are within specification with slopes between 0.9 and 1.1 and correlation coefficients, $R^2 > 0.90$.

**Table 10: Linearity of the EarlyCDT Lung test through dilution**

| Auto-antibody | Plate | Antigen at 50nM | | | Antigen at 160nM | | |
|---|---|---|---|---|---|---|---|
| | | Intercept | Gradient | $R^2$ | Intercept | Gradient | $R^2$ |
| **p53** | Plate 1 | -0.049 | 0.998 | 0.979 | -0.033 | 1.000 | 0.991 |
| **p53** | Plate 2 | -0.036 | 1.018 | 0.994 | 0.030 | 1.015 | 0.987 |
| **SOX2** | Plate 1 | -0.040 | 1.001 | 0.987 | -0.024 | 1.002 | 0.996 |
| **SOX2** | Plate 2 | -0.037 | 1.008 | 0.991 | -0.022 | 1.004 | 0.997 |
| **CAGE** | Plate 1 | -0.061 | 1.000 | 0.969 | 0.024 | 0.962 | 0.997 |
| **CAGE** | Plate 2 | -0.059 | 0.996 | 0.969 | 0.048 | 0.940 | 0.992 |
| **NY-ESO-1** | Plate 1 | -0.066 | 0.995 | 0.962 | -0.094 | 0.998 | 0.931 |
| **NY-ESO-1** | Plate 2 | -0.061 | 1.003 | 0.971 | -0.100 | 0.998 | 0.923 |
| **GBU4-5** | Plate 1 | 0.036 | 1.002 | 0.977 | 0.065 | 1.019 | 0.951 |
| **GBU4-5** | Plate 2 | 0.004 | 1.006 | 0.999 | -0.047 | 0.996 | 0.980 |
| **MAGE A4** | Plate 1 | -0.113 | 1.016 | 0.920 | -0.115 | 1.008 | 0.911 |
| **MAGE A4** | Plate 2 | -0.106 | 1.023 | 0.935 | -0.109 | 1.014 | 0.924 |
| **HuD** | Plate 1 | 0.005 | 0.993 | 1.000 | -0.022 | 0.995 | 0.994 |
| **HuD** | Plate 2 | -0.005 | 0.978 | 0.995 | -0.015 | 0.996 | 0.997 |

**Analytical Specificity, Interferences and Cross Reactions**

The effect of potential interfering substances in serum samples positive for autoantibodies measured in the EarlyCDT Lung test kit was evaluated. The following interferents tested (table 11) did not affect the performance of the assay, unless stated otherwise.

**Table 11: Interferents tested on the EarlyCDT Lung test kit**

| Interferent | Interferent test concentration* | Result |
|---|---|---|
| Triglycerides | 3274mg/dL | No significant effect p<0.01 |
| Total protein | 120g/L | Significant effect*** |
| Bilirubin (conjugated) | 20mg/dL | No significant effect p<0.01 |
| Bilirubin (unconjugated) | 20mg/dL | No significant effect p<0.01 |
| Haemoglobin | 500mg/dL | No significant effect p<0.01 |
| Albuterol | 0.4µg/mL | No significant effect p<0.01 |
| Digoxin | 6.09ng/mL | No significant effect p<0.01 |
| Rheumatoid factor | 1:1** | No significant effect p<0.01 |
| Human anti-mouse antibodies | 1:1** | No significant effect p<0.01 |

*Interferent test concentrations are those recommended by CLSI EP7-A2[19].
** An equal volume of serum positive for either RF or HAMA was added to serum positive for autoantibodies measured in the EarlyCDT Lung test kit.
*** The EarlyCDT Lung test kit should not be used in patients known to have diseases that result in an increased level of serum total protein, for example myeloma, amyloidosis, monoclonal gammopathy of undetermined significance (MGUS).

## 19. Method summary

- Equilibrate kit and all reagents to +18 to +22°C (this will take at least 2 hours).
- Prepare reagents.
- Dispense 100µL of each relevant control and diluted patient specimen into the wells of the microtitre plate(s) as described.
- Cover and incubate at +18 to +22°C for 90 minutes with shaking.
- Wash four times.
- Add 100µL of secondary antibody to all wells of the microtitre plate.
- Cover and incubate at +18 to +22°C for 60 minutes with shaking.
- Wash four times.
- Add 100µL of substrate to all wells of the microtitre plate.
- Incubate at +18 to +22°C in the dark for exactly 15 minutes (no shaking).
- Add 100µL of stop solution to all wells of the microtitre plate.
- Determine the optical density of each well at a wavelength of 650nm within 30 minutes.
- Calculate autoantibody values using the software provided on the USB device.

## 20. Glossary of terms for EarlyCDT Lung

The glossary of terms used throughout the Instructions for Use with the definitions are listed in table 12.

**Table 12: Glossary of terms**

| Term | Definition |
|---|---|
| **Accuracy** | The degree to which the result of a measurement, calculation, or specification conforms to the correct value or result. |
| **Antigen** | Immunogenic protein or other molecule. |
| **Autoantibody (AAb)** | Antibody produced in response to a host antigen. |
| **COPD** | Chronic obstructive pulmonary disease. |
| **ELISA** | Enzyme Linked Immuno-Sorbent Assay. |
| **OD** | Optical Density as measured spectrophotometrically. |
| **Pack year** | Twenty cigarettes (i.e. one pack) smoked every day for one year. |
| **PBS** | Phosphate buffered saline. |
| **Positive predictive value** | The probability that subjects with a positive test result truly have the disease. |
| **Prevalence** | The proportion of a population found to have a disease. |
| **Sensitivity** | The proportion of people known to have a disease state, who test positive for it. |
| **Specificity** | The proportion of healthy patients known not to have a disease state, who test negative for it. |

## 21. Glossary of symbols

**Manufacturer**
Indicates the medical device manufacturer.

**Consult instructions for use**
Indicates the need for the user to consult the instructions for use.

**Use-by date**
Indicates the date after which the medical device is not to be used.


**Catalogue number**
Indicates the manufacturer's catalogue number so that the medical device can be identified.

**Temperature limit**
Indicates the temperature limits to which the medical device can be safely exposed.


**Part number**
Indicates the identity of a component of the kit test

**Importer**
Indicates any natural or legal person established within the European Union that places a device from a third country on the Union market

**Irritant hazard symbol**
May cause an allergic skin reaction


***In vitro* diagnostic medical device**
Indicates a medical device that is intended to be used as an *in vitro* diagnostic medical device.

**Biological risks**
Indicates that there are potential biological risks associated with the medical device.


**Batch code**
Indicates the manufacturer's batch code so that the batch or lot can be identified.

**Do not re-use**
Indicates a medical device that is intended for one use, or for use on a single patient during a single procedure.

**Contains sufficient for <*n*> tests**
Indicates the total number of IVD tests that can be performed with the IVD kit reagents.


**Authorised representative in the European community**
Indicates the authorised representative in the European community

**Keep away from sunlight**
Indicates a component that needs protection from light sources

## 22. References


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## 23. Warranty

This product is warranted to perform as described in its labelling and literature when used in accordance with all instructions. Freenome Ltd. DISCLAIMS ANY IMPLIED WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE, and in no event shall Freenome Ltd. be liable for consequential damages. Replacement of the product or refund of the purchase price is the exclusive remedy for the purchaser.

## 24. Technical assistance and customer service

For technical assistance or to place an order, please telephone Freenome on +44 (0)115 784 0501, email earlycdt@freenome.com or see our website at www.freenome.com

## 25. Summary of Safety and Performance

The device's summary of safety and performance shall be available on EUDAMED (link: EUDAMED Web portal) when the system is fully functional.

## 26. Reporting of Serious Incidents

If a serious incident has occurred in relation to this device, it should be reported to Freenome Customer Service on +44 (0)115 784 0501 or email EarlyCDT@Freenome.com. In European Union Member States, serious incidents should also be reported to the competent authority, (the government department responsible for medical devices) in your country. Please refer to your government website for details of how to contact your competent authority.

A ‘serious incident’ means any incident that directly or indirectly led, might have led or might lead to:

- the death of a patient, user or other person,
- the temporary or permanent serious deterioration of a patient’s, user’s or other person’s state of health.

## 27. Revision history

| Version ID | Date | Summary of changes |
|---|---|---|
| V10 | Sep 2021 | Update to comply with (EU) 2017/746 IVD Regulation requirements |
| V11 | Dec 2021 | Update to Summary of clinical study results section to include additional clinical performance characteristics. |
| V12 | Jul 2022 | Addition of summary of safety and performance location. Update to shaker speeds. Additional information on buffer composition in Table 1. Recommendation to use electronic multichannel pipette for dispensing added. |
| V13 | Sep 2023 | Replacement of Oncimmune Ltd with Freenome Ltd and authorised representative updated |

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