Oncolipsy PD-L1

Manufacturer**: Pharmassist Ltd**

Instructions for Use (IFU)

PD-L1 kit

Instruction Manual

Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR) based method for the quantitative determination of Programmed death-ligand 1 (PD-L1) expression.

<24 samples>

-20°C

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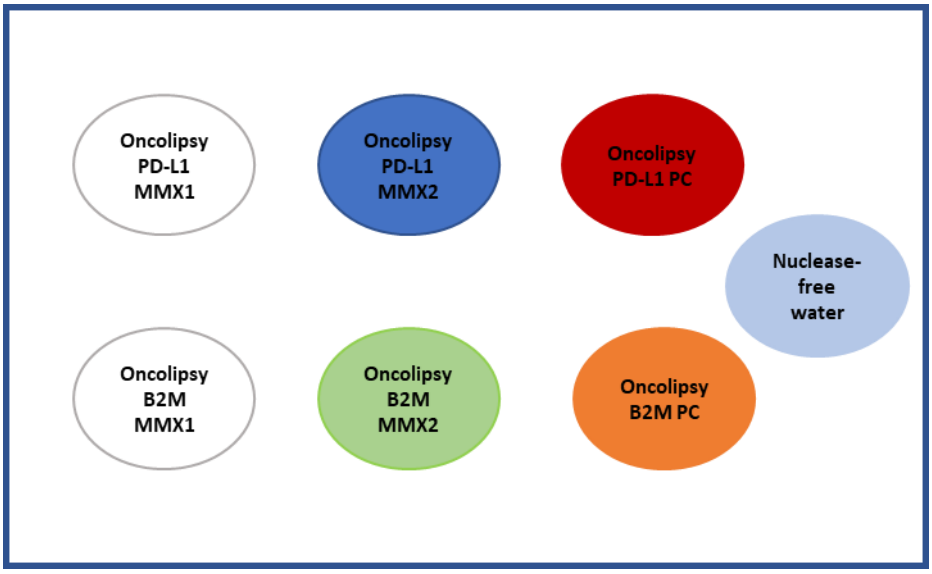
Contents

1. Kit Contents

Each Oncolipsy PD-L1 kit contains 7 vials (2 amber/dark and 5 transparent vials) with different color caps (red, green, blue, orange, light blue, and colourless) including PCR buffer master-mixes, primers/probe master-mixes, positive controls for PD-L1 and B2M biomarkers, respectively and nuclease-free water along with a leaflet. The Oncolipsy PD-L1 kit includes the following vials:

N	Kit Contents (Label)	Number of Vials	Cap Color	Volume
1	Oncolipsy PD-L1 MMX1	**1**	**Colorless cap**	600µL
2	Oncolipsy B2M MMX1	**1**	**Colorless cap**	200µL
3	Oncolipsy PD-L1 MMX2	**1**	**Blue cap**	27µL
4	Oncolipsy B2M MMX2	**1**	**Green cap**	27µL
5	Oncolipsy PD-L1 PC	**1**	**Red cap**	11µL
6	Oncolipsy B2M PC	**1**	**Orange cap**	11µL
7	Nuclease-free water	**1**	**Light Blue cap**	2mL

The Oncolipsy PD-L1 kit includes sufficient reagents to analyze 24 samples including PCR positive and negative controls. Four different RT-qPCR test runs may be performed. Each Oncolipsy PD-L1 kit contains 7 labelled vials as indicated in the following schematic visualization:



2. Intended Use

The Oncolipsy PD-L1 kit constitutes a ready-to-use kit, which is based on a reverse transcription-quantitative PCR (RT-qPCR) assay for the detection of mRNA transcripts expression of PD-L1 in RNA sample extracted from Circulating Tumor Cells (CTCs) in peripheral blood of metastatic Non-Small Cell Lung Cancer (NSCLC), Head &Neck Squamous Cell Carcinoma (HNSCC) and melanoma cancer patients. The PD-L1 Kit is intended for use by laboratory trained personnel.

3. Introduction

Immune checkpoint inhibitor monoclonal antibodies (mAbs) activate the natural human immunity against cancer, leading to spectacular anti-cancer responses and extension of patient lives. Programmed death 1 (PD-1) is an immune inhibitory receptor expressed on several immune cells, particularly cytotoxic T cells. It interacts with PD-L1 ligand, which is widely expressed on tumor cells. Blocking this interaction has been shown to be an important therapeutic target. Specific diagnostic biomarker tests have been developed that can predict patient response to an individual PD1/PD-L1 mAb, thereby assisting oncologists with brand-specific patient treatment (1, 2). Although, many immunohistochemistry assays for assessing PD-L1 expression have been developed, differences in the cell type assessed for the expression and cut-off points as thresholds are leading to different scoring schemes for the readouts (3, 4). The use of molecular assays can solve these limitations, providing more robust and specific results.

However, most PD1/PD-L1 biomarker tests developed to date may only be performed on tumor biopsies, which usually are difficult to obtain and cannot track disease progression. CTCs are considered indicators of residual disease and thus are associated with an increased risk of metastasis (5). The prognostic impact of CTCs and their central role in the metastatic cascade has been repeatedly demonstrated in many types of cancer and their molecular characterization holds a very strong potential for novel approaches in the therapeutic management of cancer patients (6). CTCs hold promise to better reflect the tumor heterogeneity compared to tissue biopsies because they originate from different tumor sites. In addition, CTCs as main liquid biopsy components could lead to important insights on how tumor cells become resistant to immune therapy because they can be analyzed longitudinally (7).

PD-L1 detection in CTCs has been studied in several types of cancer such as HNSCC (8), NSCLC (9), bladder cancer (10), breast cancer (11), and urothelial cancer (12), providing a non-invasive clinical application to predict and monitor the clinical outcome of cancer patients receiving PD-1/PD-L1 checkpoint inhibitors.

4. Kit principle

The Oncolipsy PD-L1 Kit provides all detection reagents required to determine *PD-L1* expression. The Kit provides an assay that is highly specific for *PD-L1* mRNA and is designed to completely avoid primer–dimer formation, false priming sites, formation of hairpin structures and hybridization to genomic DNA, while it amplifies specifically only *PD-L1* target gene according to the BLAST Sequence Similarity Search tool (NCBI, NIH). RT-qPCR data for *PD-*

L1 expression is normalized in respect to *B2M* expression in the cDNA samples, using the $2^{-\Delta\Delta Ct}$ approach. Each MMX2 consists of specific primers and hydrolysis probes for each target gene. Each positive control contains approximately 10^5 copies/µL of a synthetic mini-gene. Quantification is based on Real-Time monitoring during PCR amplification of fluorescently labeled hydrolysis probes that are specific for *PD-L1* and *B2M*, respectively.

The Oncolipsy PD-L1 kit is based on a reverse transcription-quantitative PCR (RT-qPCR) assay for the detection of PD-L1 mRNA transcripts expression of PD-L1 in RNA sample of Circulating Tumor Cells (CTCs) of metastatic NSCLC, HNSCC and melanoma cancer patients. The assay is developed to be compatible with Roche® LightCycler 480 and Bioer Quant Gene 9600 instruments.

4.1. CTCs Enrichment, RNA Extraction and cDNA synthesis

For the enrichment of CTCs, a size-based microfluidic device, such as Parsortix CE-marked system (ANGLE plc, UK) can be used. As for the RNA extraction and the cDNA synthesis, the validation of the assay was performed using the TRIZOL® LS reagent (ThermoFischer Scientific); and the High-Capacity RNA-to-cDNA™ Kit (ThermoFischer Scientific) respectively.

4.2. Real-Time PCR Instruments

The Oncolipsy PD-L1 kit was developed and validated to be used with the Roche® LightCycler 480 and Bioer Quant Gene 9600 instruments.

5. Analytical performance

- Dilutions ranging from 10^5 to 3 copies of PD-L1 and B2M standard per reaction have shown linearity over the entire quantification range with correlation coefficients larger than 0.99 indicating a precise log-linear relationship. LOD of both assays is 3 copies/µL and LOQ is 9 copies/µL.

- Intra-assay precision is expressed as the SD for the Cq variance in 6 replicates using three different LOT numbers of the kit. Inter-assay precision of *PD-L1* RT-qPCR assay was estimated as follows: a synthetic standard corresponding to: a) 10 copies/reaction was estimated as: Cq±SD as: 36.09±0.48 (CV% =0.34), b) 10^3 copies/reaction was estimated as: Cq±SD as: 31.52±0.25 (CV% =0.17) and c) 10^5 copies/reaction was estimated as: Cq±SD as: 24.34±0.41 (CV% =0.29). Inter-assay precision of B2M RT-qPCR assay was estimated as follows: a synthetic standard corresponding to: a) 10 copies/reaction was estimated as: Cq+SD as: 35.47+0.43 (CV% =0.31), b) 10^3 copies/reaction was estimated as: Cq+SD as: 32.30+0.50 (CV% =0.36) and c)10^5 copies/reaction was estimated as: Cq+SD as: 24.80+0.67 (CV% =0.49).

- Inter-assay precision was estimated using three different LOT numbers of the kit. Each calibrator was analyzed in 6 replicates in 5 different analytical runs. Intra-assay precision is expressed as the SD for the Cq variance. Intra-assay precision of *PD-L1* RT-qPCR assay was estimated as follows: a synthetic standard corresponding to: a) 10 copies/reaction was estimated as: Cq±SD as: 36.39±0.95 (CV% =0.74), b) 10^3 copies/reaction was

estimated as: Cq±SD as: 31.50±0.34 (CV% =0.24) and c) 10^5 copies/reaction was estimated as: Cq±SD as: 24.40±0.45 (CV% =0.32). Inter-assay precision of *B2M* RT-qPCR assay was estimated as follows: a synthetic standard corresponding to: a) 10 copies/reaction was estimated as: Cq±SD as: 35.40±1.2 (CV% =0.95), b) 10^3 copies/reaction was estimated as: Cq±SD as: 31.74±0.68 (CV% =0.50) and c) 10^5 copies/reaction was estimated as: Cq±SD as: 24.71±0.56 (CV% =0.40).

6. Identification of Material & Devices Required but Not Provided

6.1. Reagents Devices

• Appropriate CTC isolation system (Section 4.1. "CTCs Enrichment, RNA Extraction and cDNA synthesis").

a. Equipment

• Laminar Flow Hood with ultraviolet (UV) lamp
• Centrifuge
• Vortex
• White Roche® LightCycler 480 Multiwell plate 96 or strips
• Adjustable pipettes
• Pipette tips with filters
• Disposable gloves
• 0.5ml PCR microcentrifuge tubes for Section 7.1 extraction and Section 7.2 Master Mix preparation.
• ThermoCycler (for cDNA synthesis)

b. Real-Time PCR Machine

• Appropriate Real-Time PCR instrument (please refer to Section 4.2. "Real-Time PCR Instruments").

7. Protocol

7.1. Sample preparation

RNA samples will be extracted from CTCs derived from peripheral blood.

7.1.1. RNA extraction from CTCs

- For CTC analysis, 10 mL peripheral blood in EDTA from metastatic patients is required. However, the first 5 ml of blood should be discarded to avoid contamination of skin epithelial cells. Peripheral blood should be collected into K_2EDTA tubes (BD Vacutainer, Plymouth, UK) and mixed immediately after blood draw by inverting gently, maintained at room temperature (RT) and processed preferably within 3 h, to CTC isolation.

- CTCs enrichment can be performed using the Parsortix CE-marked system (ANGLE plc, UK) that is a size-based microfluidic device.
- For total RNA extraction, we recommend the following reagent from ThermoFisher Scientific (USA): the TRIZOL® LS reagent for RNA extraction step. Following RNA extraction, total RNA is suspended in 11 µL of RNase-Free Water (RNFW), and 10 µL of RNA are used for cDNA synthesis.

7.1.2 cDNA synthesis

cDNA synthesis is performed using 10µl of extracted RNA and according to manufacturer's instructions.

NOTE: Reagents for RNA extraction and cDNA synthesis are not included.

Before starting: Thaw all reagents and RNase-free water. Vortex, centrifuge quickly, and store on ice.

7.2. PCR Preparation

7.2.1 Preparation of Oncolipsy B2M PCR master mix

1. Prepare the Oncolipsy *B2M* MMX2 as shown in Table 1 according to the number of samples. Note that RNase-free water as negative control, and the positive control must always be included in each experiment run. For example, in case that one clinical sample (number of samples) should be analyzed, the user should prepare the Oncolipsy B2M MMX2 reagent for four reactions (number of reactions). The addition of an extra reaction is added to avoid eventual volume deviations due to pipetting. Therefore, 2.4µL of B2M MMX2 and 5.6µL water should be mixed in order to make a final volume of 8µL.

Table 1: Volumes of Oncolipsy B2M MMX2 reagent are based on number of samples and reactions.

Number of samples	Number of reactions	Volume of Oncolipsy B2M MMX2	Volume of nuclease-free water	Final Volume
1	4	2.4	5.6	8
2	5	3	7	10
3	6	3.6	8.4	12
4	7	4.2	9.8	14
5	8	4.8	11.2	16

6	**9**	**5.4**	**12.6**	**18**
7	**10**	**6**	**14**	**20**

2. In the above PCR tube containing Oncolipsy B2M MMX2, add 5µL of Oncolipsy B2M MMX1 and 11µL of nuclease-free water per reaction. Then vortex and collect liquid at the bottom of the tube before use. Volumes of reagents needed for Oncolipsy B2M PCR master mix are presented in Table 2.

Table 2: Preparation of Oncolipsy B2M PCR mastermix

Component	Volume/reaction (µL)
Oncolipsy B2M MMX1	5
Oncolipsy B2M MMX2	2
Nuclease-free water	11
Total volume	**18**

3. Dispense 18 µL of the Oncolipsy B2M PCR Master Mix into each reaction well of the microwell plate or strip. Do not allow the pipettor tip to touch the plate outside the well.
4. Pipette 2 µL of nuclease-free water into B2M negative control (NC) of the microwell plate or strip; mix well using pipette to aspirate and dispense within the well a minimum of two times.
5. Pipette 2 µL of Oncolipsy B2M PC into well of the microwell plate or strip; mix well using pipette to aspirate and dispense within the well a minimum of two times.
6. Using new pipettor tips for each cDNA sample, add 2 µL of the first cDNA sample to well of the microwell plate or strip; mix each well using a pipette to aspirate and dispense within the well a minimum of two times. Repeat this procedure for all cDNA samples.
7. Cover the microwell plate or strip with sealing film or caps, respectively.
8. Confirm that all liquid is collected at the bottom of each well before starting PCR.
9. Place the plate or strip in the instrument and start the run according to the protocol below.

7.2.2 Preparation of Oncolipsy PD-L1 PCR master mix

1. Prepare the Oncolipsy PD-L1 MMX2 into a 0.5mL PCR tube as shown in Table 3 according to the number of samples. Note that RNase-free water as negative control and the PC control must always be included in each experiment run. For example, in case that one clinical sample (number of samples) should be analyzed, the user should prepare the Oncolipsy PD-L1 MMX2 for four reactions (number of reactions). Therefore, 2.4µL of Oncolipsy PD-L1 MMX2 and 5,6µL water should be mixed in order to make a final volume of 8µL.

Table 3: Volumes of Oncolipsy PD-L1 MMX2 are based on number of samples and reactions.

Number of samples	Number of reactions	Volume of Oncolipsy PD-L1 MMX2	Volume of nuclease-free water	Final Volume
1	4	2.4	5.6	8
2	5	3	7	10
3	6	3.6	8.4	12
4	7	4.2	9.8	14
5	8	4.8	11.2	16
6	9	5.4	12.6	18
7	10	6	14	20

2. In the above PCR tube containing Oncolipsy PD-L1 MMX2, add 15µL of Oncolipsy PD-L1 MMX1 and 1µL of nuclease-free water per reaction. Then vortex the tube and collect liquid at the bottom of the tube before use. Volumes of reagents needed for Oncolipsy PD-L1 PCR master mix are presented in Table 4.

Table 4: Preparation of Oncolipsy PD-L1 PCR master mix

Component	Volume/reaction (µL)
Oncolipsy PD-L1 MMX1	15
Oncolipsy PD-L1 MMX2	2
Nuclease-free water	1
Total volume	**18**

3. Dispense 18µL of the PD-L1 PCR Master Mix into each reaction well of the microwell plate or strip. Do not allow the pipettor tip to touch the plate outside the well.
4. Pipette 2µL of water into PD-L1 negative control (NC) of the microwell plate or strip; mix well using pipette to aspirate and dispense within the well a minimum of two times.
5. Pipette 2µL of Oncolipsy PD-L1 PC into well of the microwell plate or strip; mix well using pipette to aspirate and dispense within the well a minimum of two times.
6. Using new pipettor tips for each cDNA sample, add 2 µL of the first cDNA sample to well of the microwell plate or strip; mix each well using a pipette to aspirate and dispense within the well a minimum of two times. Repeat this procedure for all cDNA samples.
7. Cover the microwell plate or strip with sealing film or caps, respectively.
8. Confirm that all liquid is collected at the bottom of each well before starting PCR.

9. Place the plate or strip in the instrument and start the run according to the protocol below.

7.2.3 Programming the Real-Time PCR Instrument

Enter the following PCR cycling program for B2M RT-qPCR:

Step	Temperature	Time	Cycles
Denaturation	95°C	2 min	1
Denaturation	95° C	10 sec	45
Annealing	58° C	20 sec	
Extension	72° C	20 sec	
Cooling	40° C	30 sec	1

Enter the following PCR cycling program for PD-L1 RT-qPCR:

Step	Temperature	Time	Cycles
Denaturation	95°C	2 min	1
Denaturation	95° C	10 sec	55
Annealing	58° C	20 sec	
Extension	72° C	20 sec	
Cooling	40° C	30 sec	1

NOTE: Please select the following detection format for FAM: Mono Color Hydrolysis probe/UPL probe for LightCycler 480 (Roche® Diagnostics) and Bioer Quant Gene 9600 instruments.

8. Results

Note: A test run in order to be valid should meet the following criteria:

- In NC no signal should be detected
- The B2M PC value must range between 23.00-25.60 and PD-L1 PC value must range between 21.80-25.30
- The B2M Cq value of clinical samples must be below 30.

9. Interpretation of results

qPCR data for *PD-L1* expression should be normalized in respect to *B2M* expression in the same cDNAs, using the $2^{-\Delta\Delta Ct}$ approach.

For CTC analysis use the equation *

$$2^{-\frac{(Cq_{PD\text{-}L1}-Cq_{B2M})}{11.95}}$$

*: The above equation is derived from the analysis of 40 healthy donor (HD) individuals analyzed in exactly the same way as clinical samples. This equation reported for CTCs should be only used when exactly the above-mentioned procedure (4.1) for RNA extraction and cDNA synthesis is used. In any other case at least 10 samples from healthy donors should be analysed in exactly the same way as clinical samples and this cut-off should be re-evaluated. In order to characterize a sample as positive for PD-L1 mRNA overexpression the value of the above equation should be over 2.49.

10. Shipping, Storage & Handling Conditions

The Oncolipsy PD-L1 kit is shipped at a styrofoam box, which contains dry ice and must be stored in a constant-temperature freezer at -20°C upon arrival.

Oncolipsy PD-L1 kit is stable until the stated expiration date when stored under the specified storage conditions. Reagents can be stored in their original packaging at –20°C for 18 months, until the stated expiry date shown on the packaging.

The components must not be used beyond the expiration date shown on the packaging. Repeated thawing and freezing should be avoided. Do not exceed a maximum of five freeze – thaw cycles.

If the protective kit packaging is damaged upon receipt, please contact Pharmassist for instructions.

11. Warning and Precautions

11.1. Handling and Procedural Requirements

• Always wear disposable gloves when handling kit components.

• Use separated working areas for specimen preparation, reaction set up and amplification.

• Supplies and equipment should be separated in each work area and not moved between them.

• When mixing reagents by pipetting up and down this should be done with a volume roughly equal to 50% of the total component volume.

11.2. Precautions

To reduce risk of contamination:

- RNA extraction, cDNA synthesis, preparation of the Real-Time PCR steps and thermocycling should be performed in separate rooms.
- Preparation of the PCR mixture should be done in a PCR-hood.

- In every extraction or synthesis step during the whole procedure, filter tips, RNAse-DNAse-free reaction vials and calibrated precision pipettes dedicated for each step should be used.
- Positive controls should be opened and processed away from test samples and kit components to avoid cross-contamination.
- Before each procedure, PCR hood and the acquired equipment (filter tips, reaction vials, pipettes etc.) should be disinfected with UV for 20 minutes. The entire hood work surface, as well as pipettes, should be clean using 10% bleach prior to UV.
- Use DNase/RNase free plasticware and pipettes reserved for DNA/RNA work to avoid the cross-contamination from shared equipment.
- Throughout the whole procedure use DNase/RNase free filter tips to avoid further contamination.
- Do not mix vials from different kits, even if they are produced in the same lot.

12. Limitations of Use

• The Oncolipsy PD-L1kit has been validated for use with CTC samples run on the Roche® LightCycler 480 Real-Time PCR and Bioer Quant Gene 9600 instruments.

• The procedures in this handbook must be followed, as described. Any deviations may result in assay failure or cause erroneous results.

• Good laboratory practice is required to ensure the performance of the kit, with care required to prevent contamination of the kit components. Components should be monitored for contamination and any components thought to have become contaminated should be discarded as standard laboratory waste in a sealed pouch or zip-lock plastic bag.

• All samples should be handled as if they are infectious following proper biosafety precautions.

• Interpretation of results must account for the possibility of false negative and false positive results.

• False negative results may be caused by:

  - Pre-analytical errors, eg unsuitable sample collection, handling and/or storage of samples.
  - Failure to follow procedures in this handbook.
  - Use of unauthorised extraction kit or PCR platform.

• False positive results may be caused by:

  - PCR contamination

• All results should be interpreted by a health care professional in the context of patient medical history and clinical symptoms.

13. Explanation of Symbols

Symbol	Explanation
	Contains sufficient for <n> samples
	In vitro diagnostic use
	In vitro Diagnostic Medical Device
	Batch Code
	Limit of Temperature
	Manufacturer

14. References

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