MAXIMIZING REPRODUCIBILITY AND SENSITIVITY IN QPCR FOR DETECTING TRANSCRIPTS OVER A BROAD DYNAMIC RANGE IN RESPONSE TO ANTI-PD-1 THERAPY

David Good, Melodie Boute, Corinne Ramos & Mathieu Gaudin

Aliri France SAS, 152 Rue du Dr Yersin, 59120 Loos, France

CONTACT INFORMATION: info@aliribio.com

#T1530-01-02

PURPOSE

This study aims to optimize and validate a qPCR workflow for the reproducible and sensitive quantification of immune checkpoint transcripts (PD-1, PD-L1, CTLA-4) in FFPE lung cancer tissues. By refining tissue preparation, RNA input, and assay conditions, we establish a robust method for detecting gene expression across a broad dynamic range, enabling reliable assessment of immunotherapy response and supporting biomarker-driven patient stratification.

METHOD(S)

Sample Preparation

Formalin-fixed, paraffin-embedded (FFPE) lung tumor tissues from NSCLC patients treated with pembrolizumab were selected based on known clinical response. Tissue integrity and surface area were assessed via HE staining and digital scanning to ensure comparability across samples.

RNA Extraction and Quality Control

Total RNA was extracted using the MagMax kit, incorporating xylene deparaffinization, ethanol washes, proteinase K digestion, DNase treatment, and magnetic bead purification. RNA purity and concentration were measured with Nanodrop, and integrity was confirmed using Bioanalyzer 2100.

Reverse Transcription and qPCR

500 ng of RNA was reverse-transcribed using the High-Capacity cDNA Kit. qPCR was performed on a QuantStudio 7 Pro targeting PD-1, PD-L1, and CTLA-4, with GAPDH as the reference gene. No-template and no-RT controls were included. Data were analyzed using the $2^{-\Delta\Delta Cq}$ method, and statistical significance was assessed via Mann–Whitney tests (p-value < 0.05).

RESULTS

1. Tissue Preparation and RNA Yield Optimization

Surface area analysis of FFPE lung tumor sections revealed variability in RNA yield depending on sectioning conditions. **Table 1** summarizes the measured surface areas of five NSCLC tissue samples, highlighting differences that impact RNA recovery and normalization strategies

The optimal protocol—5 sections at 8 µm—provided high RNA concentration (154.4 ng/µL) with acceptable purity (A260/280 = 2.05; A260/230 = 1.39), ensuring suitability for downstream qPCR (**Table 2**).

2. qPCR Efficiency and Linearity

Standard curves for PD-1, PD-L1, and CTLA-4 demonstrated high linearity ($R^2 > 0.99$) and efficiency (90–110%), validating the assay's accuracy across a broad dynamic range of input cDNA (**Figure 1**).

3. Reproducibility Across Experiments

Intra- and inter-day reproducibility was confirmed, with 87.5% of CV values below 10% for PD-1, PD-L1, and CTLA-4 for intra-day-RT-qPCR and 100% of CV values below 10% for inter-day-RT-qPCR (**Figure 2**). These results support the robustness of the qPCR setup for consistent immune marker quantification across samples and timepoints.

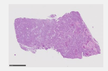
4. Sensitivity to cDNA Input

Reliable amplification was achieved across a wide range of cDNA inputs (1–50 ng). Inputs ≥10 ng yielded consistent expression with minimal variability, establishing a recommended minimum for accurate quantification, especially in low-yield samples (**Figure 3**).

5. Transcript Expression and Therapy Response

Comparative analysis between responder and non-responder NSCLC samples revealed significant upregulation of PD-1 (×4.5), PD-L1 (×52), and CTLA-4 (×7) in the responder (**Figure 4**), suggesting potential utility of these markers in predicting immunotherapy outcomes.

Tissue	1	2	3	4	5
Area (mm²)	53.6	20.1	12.2	64.3	43.5



Tissue 1

Table 1: Tissue Sample Surface Analysis. HE-stained scans of lung tumor sections (NSCLC) were analyzed to measure the surface area of each sample. Surface measurements for the five tissue samples were obtained using the NDP.view2 software.

Conditions	Starting Material (µm)	Concentration (ng/µL)	A260/A280	A260/A230
5 sections (6µm)	30	141.7	2.06	1.51
5 sections (8µm)	40	154.4	2.05	1.39
8 sections (6µm)	48	97.8	2.04	1.09
8 sections (8µm)	64	104.2	2.04	1.13
10 sections (6µm)	60	102.2	2.05	1.52
10 sections (8µm)	80	251.1	2.06	1.6

Table 2: Assessment of nucleic acid extraction from tissue samples under different sectioning conditions. The parameters evaluated include the number and thickness of tissue sections, the initial amount of material, the concentration of extracted nucleic acids, and purity ratios (A260/A280 and A260/A230). Data were obtained following quality control with the Nanodrop One.

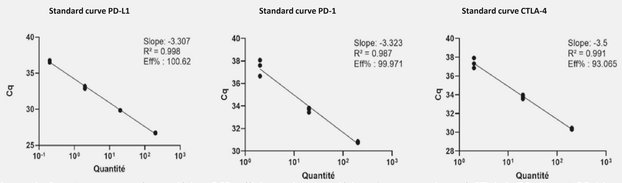


Figure 1: Standard curves for verifying PCR efficiency in quantifying the expression of CTLA-4, PD-1, and PD-L1 genes. Standard curves are generated by plotting threshold cycle (Ct) values against the logarithm of input quantities. Key parameters, including the slope, coefficient of determination (R^2), and PCR efficiency (Eff%) are provided for each target gene.

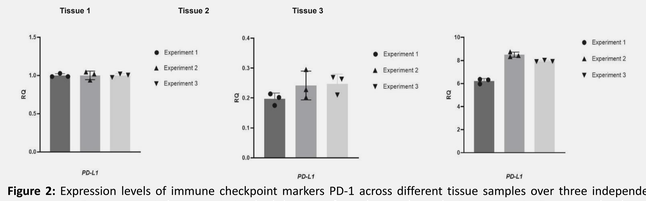


Figure 2: Expression levels of immune checkpoint markers PD-1 across different tissue samples over three independent experiments. Bars represent the mean ± standard deviation from three independent experiments. Expression levels were normalized to GAPDH and referenced to a calibrator. Expression levels of each marker were then compared between experiments within the same tissue sample.

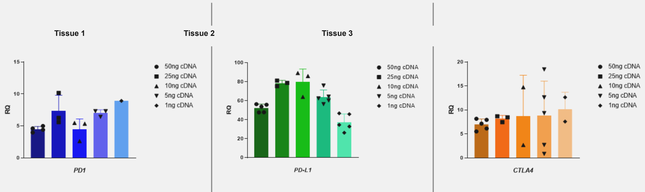


Figure 3: Sensitivity of PD-1, PD-L1, and CTLA-4 probes relative to cDNA input and its impact on immune marker expression. The graphs display the relative expression levels (RQ) of PD-1 (left), PD-L1 (center), and CTLA-4 (right) as measured by qPCR. Different cDNA input amounts were tested: 50 ng (■), 25 ng (▲), 10 ng (▼), 5 ng (◆), and 1 ng (●). Each bar represents the mean ± standard deviation of three technical replicates for 10 and 25 ng, and five technical replicates for 50, 5, and 1 ng.

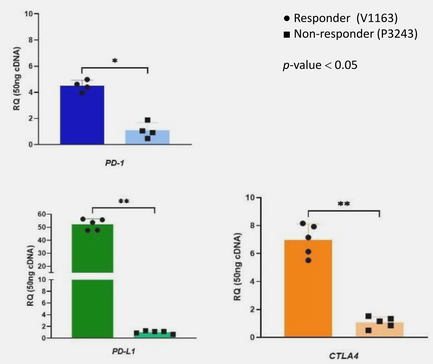


Figure 4: Comparative analysis of immune marker gene expression (PD-1, PD-L1, and CTLA4) in NSCLC lung cancer samples with known responses to pembrolizumab immunotherapy. Sample V1163_Tp14 is a responder, while sample P3243_Tp14 is a non-responder to immunotherapy. The graphs display the relative expression levels (RQ) of PD-1 (left), PD-L1 (center), and CTLA-4 (right) in samples V1163_Tp14 and P3243_Tp14, as measured by qPCR. Expression levels for each marker were normalized to GAPDH and referenced to a calibrator. Each bar represents the mean ± standard deviation of five technical replicates. A Mann-Whitney test was performed, with values considered significant at p > 0.05.

CONCLUSION(S)

This work successfully optimized qPCR conditions for the reproducible and sensitive quantification of immune markers PD-1, PD-L1, and CTLA-4 in lung cancer tissue. By establishing reliable assay conditions, we ensured consistent detection across different tissue types and identified the optimal cDNA input to balance sensitivity with minimal sample usage. These findings provide a robust framework for using qPCR to assess immune marker expression, offering insights that could enhance the evaluation of patient responses to immunotherapy.