ANGLE

CELLS FOR PRECISION MEDICINE.

Application Note

Downstream RNA Workflow: Extraction, Reverse Transcription, and Real-Time PCR

Introduction

The procedures described in this document are for RESEARCH USE ONLY and not for use in clinical or diagnostic procedures.

Detection and characterisation of circulating tumour cells (CTCs) can be carried out by assessing the expression level of target genes in the blood sample.

This Application Note describes procedures for messenger RNA analysis of CTCs separated by the Parsortix™ instrument. In what follows, detection of overexpression of tyrosinase and the melanoma associated antigen, gp100, have been used, as an illustrative example, to identify spiked melanoma cells after enrichment from blood using the Parsortix instrument. Use of alternative reagents and methods to detect different targets will need to be optimised and validated by the end-user.

The workflow contains the following steps:

- cell lysis and RNA extraction
- reverse transcription
- real-time PCR.

Reagents and Consumables

Reagents

Reagents	Catalogue Number	Manufacturer
2-Mercaptoethanol	M6250	Sigma Aldrich
RNeasy Micro Kit (50)	74004	Qiagen
Transcriptor Universal cDNA Master	05893151001	Roche
TaqMan® Universal PCR Master Mix	4364338	Life Technologies

Single Tube TaqMan® Gene Expression Assays from Life Technologies

Target Gene	Catalogue Number	Manufacturer
Tyrosinase	HS01099965_M1	Life Technologies
PMEL (GP100)	HS00173854_M1	Life Technologies
Beta-2 Microglobulin	HS00187842_M1	Life Technologies

Note that these genes are included for proof of principle of the workflow, and are not intended to be used as a diagnostic procedure to identify the presence of CTCs in a blood sample separated using the Parsortix system.

Methods

Parsortix System Procedure

1. Following the instructions in the user manual (Parsortix PR1 Information for Users), separate 10 ml of whole blood using a 6.5 µm cassette and harvest cells in 210 µl into a clean RNase free 1.5 ml Eppendorf tube.

Cell Lysis and RNA Extraction

1. Add 400 µl RNA lysis buffer (RLT from RNeasy Micro Kit, Qiagen) containing 1% 2-Mercaptoethanol to the 210 µl cell harvest.
2. Vortex the tube vigorously for 1 minute to homogenize the sample. At this stage, the cell lysate can be stored at -80°C, otherwise, extract RNA using the manufacturer's instructions for the RNeasy Micro Kit, Qiagen. Remember to adjust the volume of 70% ethanol accordingly.
3. Elute RNA in 15 µl water.

Reverse Transcription

1. Follow the manufacturer's instructions for the Transcriptor Universal cDNA Master kit (Roche) to reverse transcribe the entire 15 µl RNA sample.
2. In a micro-tube combine the following, mix and briefly spin down:

   4 µl Transcriptor Universal Reaction Buffer (vial 2)

   1 µl Transcriptor Universal Reverse Transcriptase (vial 1)

   15 µl RNA.
3. Run the following thermal profile:

   25°C for 5 minutes

   55°C for 10 minutes

   85°C for 5 minutes

   hold at 4°C.

Real-time PCR

1. To a new PCR tube/well add the following:

   5 µl 2X TaqMan® Universal PCR Master Mix

   0.5 µl specific Life Technologies primer/probe mix

   3.5 µl water

   1 µl undiluted cDNA.

2. Run the following thermal profile (LightCycler® 480, Roche was used in development and tests of these procedures):

   50°C for 2 minutes

   95°C for 10 minutes

   50X { 95°C for 15 seconds
   60°C for 1 minute (single acquisition) }

   Hold at 4°C.

Sample Outcomes

- The housekeeping gene, beta-2 microglobulin, must be positive for results to be valid.
- Results are considered positive if an amplification curve is present and negative if no amplification is detected.
- Example of results using LightCycler® 480:

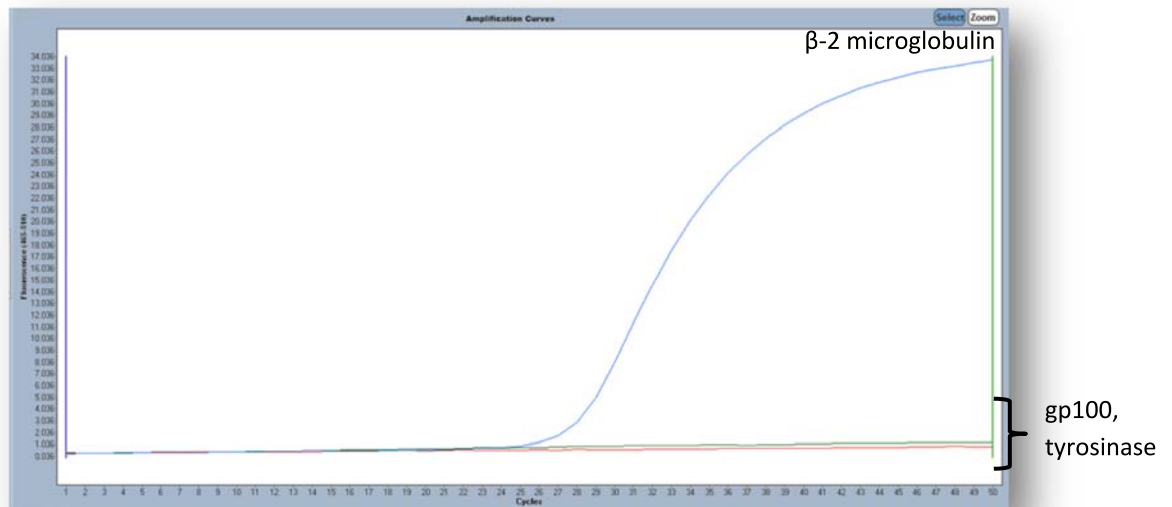

Figure 4.1 Amplification curves for an un-spiked blood sample. *PCR results for a blood sample from a healthy normal volunteer is negative for both tyrosinase and gp100 and positive, as expected, for the housekeeping gene, beta-2 microglobulin.*

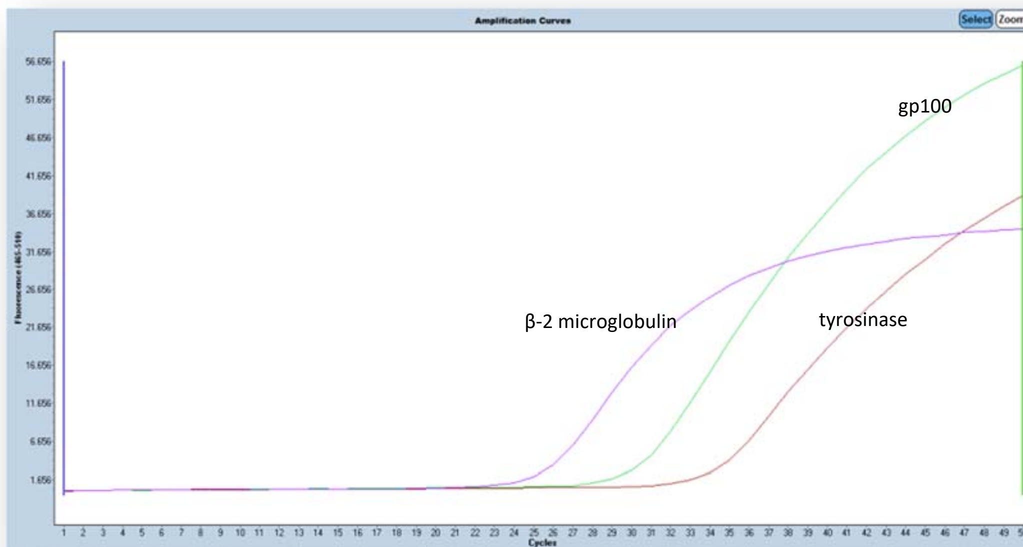

Figure 4.2 Amplification curves for a blood sample spiked with 10 x SKMEL-28 cells. *SKMEL-28 cells are known to overexpress tyrosinase and gp100. The RNA analysis of a blood sample spiked with 10 SKMEL-28 cells by PCR shows an amplification of tyrosinase and gp100, in addition to the housekeeping gene, beta-2 microglobulin.*

Specificity

Assessment of specificity was performed by separating 18 unspiked blood samples obtained from 8 normal healthy volunteers over two days. All samples showed no amplification for the target genes gp100 and tyrosinase whilst the house keeping gene (beta-2 microglobulin) was positive with an average ct value of 24.4.

Sensitivity

- Evaluation of sensitivity was performed by spiking 10 SKMEL-28 cells (pre-labelled with CellTracker Green, Life Technologies) into 18 whole blood samples obtained from healthy normal volunteers. Each of the 10 ml samples was separated and captured cells were counted using a fluorescence microscope prior to harvesting.
- The average number of captured cells was 8 cells across all 18 samples with the highest number being 20 and the lowest being 4 cells.
- The PCR results showed an amplification of gp100 in all 18 samples with an average ct value of 30.6, demonstrating 100% sensitivity. 16 of the samples showed positive results for tyrosinase with an average ct value of 33.8, a sensitivity of 88.9%.
- The housekeeping gene was positive for all 18 samples, with an average ct value of 22.9.

Technical Notes

Setting up the technique

- The end-user should identify and carefully optimise the RNA panel of interest. It is essential to assess the background expression of the target gene/s in contaminating white blood cells. It is also crucial to take into consideration the variabilities in the number of residual white blood cells in the harvest. This can be due to normal biological variations in the leukocytes count between donors/patients, or due to technical variabilities during processing.
- It is important to verify the successful and reliable working of this technique before it is deployed experimentally. The best way to do this is to run the procedure on spiked fluorescently pre-labelled cells that can be traced in the cassette to confirm the capture of the cells and to establish the sensitivity of the assay.

Interpretation of the results

- Care is required when detection of mRNA in the isolated cells is used to assess the presence/absence of CTCs. Due to the transcriptionally heterogeneous nature of cancer cells, which might result in the presence of isolated cancer cells that do not necessarily express the expected target gene to a detectable level, a negative RNA result does not necessarily mean the non-existence of CTCs in the harvest. Therefore, negative RNA results can be explained by the absence of CTCs in the sample, the absence of overexpressed targets in the cells, or the insufficient enrichment of CTCs. Running appropriate controls for each experiment can help interpret negative results.
- In some circumstances, additional confirmation of the presence of CTCs can be made possible by combining RNA analysis with immunofluorescence staining. This can be done by separating an additional 10 ml of blood from the same patient at the same time point. This will also enable the enumeration of CTCs in the sample (this RNA workflow is not a quantitative method).

Additional operational points

- The age of blood and the time from separation to lysis might affect the efficiency of CTCs separation and the stability of RNA. We recommend, where possible, processing blood samples within 48 hours of collection, and lysing the cells immediately after separation. The cell lysate can be stored long-term at -80°C before the RNA is extracted. As soon as the RNA is extracted, we recommend proceeding to the reverse transcription step immediately.
- The incorporation of a cDNA pre-amplification step before the PCR using commercially available pre-amplification kits may help increase the sensitivity of the PCR to detect targets that show inefficient PCR amplification. This will need further optimisation by the end-user.

Troubleshooting

Instrument Troubleshooting

See Parsortix™ User Manual for separation, harvesting or general instrument troubleshooting.

Contact ANGLE technical support at us-support@angleplc.com or eu-support@angleplc.com for additional troubleshooting.