Jul 19, 2019

GEP analysis of BM CD34+/lin- cells of patients with CML

PLOS One

Alessandra Trojani[1]

[1]ASST Grande Ospedale Metropolitano Niguarda, Milano, Italy

1 Works for me dx.doi.org/10.17504/protocols.io.yncfvaw

Alessandra Trojani

ABSTRACT

Protocols. Trojani A. et al..doc

EXTERNAL LINK

https://doi.org/10.1371/journal.pone.0218444

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Wide-transcriptome analysis and cellularity of bone marrow CD34+/lin- cells of patients with chronic-phase chronic myeloid leukemia at diagnosis vs. 12 months of first-line nilotinib treatment. Trojani A, Pungolino E, Rossi G, D'Adda M, Lodola M, Di Camillo B, Perego A, Turrini M, Orlandi E, Borin L, Iurlo A, Malato S, Spina F, Latargia ML, Lanza F, Artale S, Anghilieri M, Carraro MC, De Canal G, Morra E, Cairoli R. Cancer Biomark: 2017 Dec 12;21(1):41-53. doi: 10.3233/CBM-170209.

GUIDELINES

Follow the guidelines indicated in the protocols including those regarding the manufacturer's instructions

MATERIALS TEXT

materials.doc

SAFETY WARNINGS

Play attention to contamination during the experiments (RNA and cDNA) ad respect the times indicated in the protocol

BEFORE STARTING

Be sure to have all the reagents and the instruments required for the experiments of the protocol

1 Automated isolation of bone marrow CD34+/lin- using immunomagnetic beads

2 1 Isolate Mononuclear cells (MNCs) from the bone marrow (BM) blood samples (range, 1-25 ml) using Ficoll density gradient centrifugation at 800 rpm for 20 minutes.

3 2 Immediately after, select the BM CD34+/lin- cells using Diamond CD34 Isolation kit and autoMACs Pro separator (Miltenyi Biotech) according to the manufacturer's instructions.

4 3 Label MNCs with a mix of biotin-conjugated antibodies against lineage-specific antigens.

5 4 Immediately after, label the cells with Anti-Biotin Microbeads.

6 5 Select the lineage-negative stem and progenitor cells by the depletion of the magnetically labeled cells. BM CD34+/lin- cells were obtained from the lineage-negative stem and progenitor cells using CD34 Microbeads (Miltenyi Biotec) and following the manufacturer's instructions.

7 Counting of BM CD34+/lin- cells and cell cryopreservation

8 6 Dilute the cell samples in Trypan blue and count the cells using hemocytometer.

9 7 Calculate the number of cells for each sample using the following formula: Total cells counted x dilution factor/n°squares x 104 cells / ml x 2 ml cell suspension.

10 8 Resuspend the cells in 50 µl of RNAlater (Thermo Fisher Scientific, Milano, Italy) and store at -20°C.

11 9 Preserve the BM CD34+/lin- cells in 50 µl of RNAlater when the number of the cell count was equal to 10,000 or more and the cells were stored at -20°C.

12 10 If the number of BM CD34+/lin- cells was less than 10,000, resuspend the cells in 10 µl of Prelude direct Lysis module (NuGEN, AC Leek, The Netherlands) and immediately after, store the cells at -80°C.

13 FISH analysis

14 11 Test BM CD34+/lin- cells by standard FISH.

15 12 Perform FISH analysis of a small sample of selected CD34+/lin- cells (containing at least 103 cells fixed in Carnoy's solution) using standard methods.

16 13 Cohybridize samples to XL BCR/ABL1 plus Translocation/Dual Fusion Probe (MetaSystems, Milan, Italy) on ThermoBrite Statspin Model (Leica Biosystems, US).

17 14 Perform FISH analysis using fluorescence microscope Axioskop 2 (Carl Zeiss Microimaging GmbH, Göttingen, Germany), equipped with a UV 100-W lamp (Osram, Augsburg, Germany), ProgRes MF CCD camera (Jenoptik AG, Jena, Germany), and ISIS System Software (MetaSystems Hard & Software, Althlussheim, Germany).

18 15 Count, at least, 200 interphase nuclei from each suitable specimen (optimum: 300 nuclei).

19 16 Read each available interphase nucleus even in sub-optimal specimens.

20 17 Obtain results according to the International System for Human Cytogenetic Nomenclature (ISCN) [24].

RNA extraction

18 Isolate total RNA from the BM CD34+/lin- cells of CML patients previously stored in RNAlater.

19 Perform RNA extraction using MagMAX 96 Total RNA Isolation Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

20 Measure quality and yield of the extracted RNA using Nanodrop (Thermo Fisher Scientific).

21 Do not perform RNA extraction of the BM CD34+/lin- cells stored in Prelude direct Lysis module (NuGEN) because of the too low number of cells and perform the RNA processing starting from the cells.

GEP experiments

22 Prepare cDNA starting from the previously extracted RNA (50 ng) using Ovation Pico WTA System V2 kit (NuGEN) and Encore Biotin Module Kit (NuGEN) following the manufacturer's instructions.

23 Hybridize cDNA to Affymetrix HTA 2.0 using the GeneChip platform (Affymetrix).

24 Use Affymetrix GeneChip Scanner 3000 according to the manufacturer's instructions to scan the signals.

25 Prepare cDNA starting from 3 µl of the BM CD34+/lin- cells resuspended in Prelude direct Lysis module (NuGEN), using Ovation One Direct System kit followed by Encore Biotin Module Kit (NuGEN) adhering to the manufacturer's instructions.

26 Hybridize cDNA to Affymetrix HTA 2.0 using the GeneChip platform (Affymetrix).

Bioinformatic analyses of GEP data

27 The preprocessing of microarray raw data was performed using R software version 3.4.2. The Affymetrix HTA 2.0 probes were initially summarized into probe sets specific for a given gene using function RMA of R package oligo, downloaded from Bioconductor repository version 3.4. Principal component analysis (PCA) has been performed using prcomp function of package stats version 3.4.2.

28 MvA plots were generated using custom scripts. MvA plots show the relationship among the average log intensity of the gene expression (A value) and the log of intensity ratio (M value) between two samples.

29 PCA and MvA plots were examined before and after microarray preprocessing as quality checking procedure. PCA plots revealed the presence of batch effects due to the different protocols used for performing RNA extraction and GEP experiments. Batch effects have been corrected using function ComBat of R package sva.

30 MvA plots showed the presence of bias in the distribution of intensities among samples, then data have been normalized using function normalize.quantiles of R package preprocessCore.

37 31 The differential expression analysis was performed on the samples at 12 months vs. diagnosis using the two-classes SAM test, implemented in the homonym function in R package samr. Benjamini-Hochberg procedure was applied to control the False Discovery Rate (FDR) and a cut-off value of 0.05 was applied to select for significant differential expression.

38 32 Functional clustering was performed on significant differentially expressed genes using online tool DAVID (https://david.ncifcrf.gov/), to classify them into functional groups based on their annotation term co-occurrence.

39 33 Use for the analysis protein coding genes having a unique EntrezID in the “Affymetrix NetAffx” annotation were used (HTA 2.0 Transcript Cluster Annotations, Release 36, 7/6/16).