Jun 29, 2021

Targeted absolute transcript quantification in single cells after whole transcriptome amplification

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dx.doi.org/10.17504/protocols.io.389grz6

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ABSTRACT

Gene expression analysis of rare or heterogeneous cell populations such as disseminated cancer cells (DCCs) requires a sensitive method allowing reliable analysis of single cells. Therefore, we developed and explored the feasibility of a quantitative PCR (qPCR) assay to analyze single-cell cDNA pre-amplified using a previously established whole transcriptome amplification (WTA) protocol. We carefully selected and optimized multiple steps of the protocol, e.g. re-amplification of WTA products, quantification of amplified cDNA yields and final qPCR quantification, to identify the most reliable and accurate workflow for quantitation of gene expression of the *ERBB2* gene in DCCs. We found that absolute quantification outperforms relative quantification. We then validated the performance of our method on single cells of established breast cancer cell lines displaying distinct levels of HER2 protein. The different protein levels were faithfully reflected by transcript expression across the tested cell lines thereby proving the accuracy of our approach. Finally, we applied our method on patient-derived breast cancer DCCs. Here, we were able to measure *ERBB2* expression levels in all HER2-positive DCCs. In addition, we could detect *ERBB2* transcript expression even in HER2-negative DCCs, suggesting post-transcriptional mechanisms of HER2 loss in anti-HER2-treated DCCs. In summary, we developed a reliable single-cell qPCR assay applicable to measure distinct levels of *ERBB2* in DCCs.

DOI

dx.doi.org/10.17504/protocols.io.389grz6

PROTOCOL CITATION

Franziska Durst, Ana Grujovic, Iris Ganser, Martin Hoffmann, Peter Ugocsai, Christoph A. Klein, Zbigniew T. Czyż 2021. Targeted absolute transcript quantification in single cells after whole transcriptome amplification. **protocols.io** https://dx.doi.org/10.17504/protocols.io.389grz6

KEYWORDS

qPCR, single cell transcriptome analysis, absolute quantification, gene expression analysis, sensitivity

CREATED

Jun 16, 2019

LAST MODIFIED

Jun 29, 2021

PROTOCOL INTEGER ID

24577

Single cell isolation

1 Single cells are manually isolated with a micromanipulator (Eppendorf, PatchMan NP2) as previously described (Hartmann CH, Klein CA. Nucleic Acids Res. 2006;34(21):e143). Isolated cells were picked in 1 µl of 1× PBS and transferred into 4.4 µl of lysing buffer containing 4 µl mTRAP™ Lysis Buffer (Active Motif) and 0.4 µl (10 ng) tRNA from E. coli MRE 600 (Roche). Picked cells suspended in the lysis can be stored at -80°C until further processing.

Whole Transcriptome Amplification

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Whole Transcriptome Amplification.docx

2.1

PNA mix (prepare before thawing the samples)

- Prepare 19 µl lysis buffer + 1 µl protease solution
- Create master mix containing equal volume of lysis buffer/protease and PNAs (n µl lysis buffer / protease 1:20 + n µl PNA), n is number of samples
- Get samples from the -80 ° C cabinet
- Add 2 µl of prepared mix to each sample to the upper fluid level (do not completely immerse) or add to the margin and centrifuge briefly
- Reagent Control: 5 µl Lysis Buffer + 2 µl PNA Mix
- Cycler program: Lysis (volume: 10 µl)

Program: **Lysis**			
	Temperature	Time (h:min:sec)	
Step 1	45.0°C	00:10:00	Protein digestion
Step 2	75.0°C	00:01:00	Degradation of the secondary structure of the mRNAs
Step 3	22.0°C	00:10:00	Binding of the PNAs
Step 4	22.0°C	forever	

- Resuspend streptavidin beads and add 4 µl beads per tube
- Place all Tubes in a 50 ml tube, fix with paper towels and roll for 45 min at RT

2.2 Preparations for RNA isolation

- Preheat the hybridization oven incl. Glass tubes to 44 ° C
- Thaw washing buffer and reagents for cDNA synthesis
- Prepare a new set of Tubes (Axygen) for supernatants, label and deliver each 0.8 µl polyacrylamide carrier (PAA carrier)
- Prepare and label a new set of Tubes (Axygen) for changing during the Tween wash step

Prepare cDNA synthesis mix I and II before starting the washing steps and store on ice (Mix II prepare without enzyme and add enzyme shortly before use)

RNA isolation

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2.3 Careful resuspension of the beads during the washing steps is critical to the quality of the genomic DNA recovered from the supernatants.
The beads must not dry out during washing, therefore remove the supernatant from maximum four samples and resuspend beads in new buffer and then process the next four samples, etc.

- Centrifuge tubes briefly
- Add 10 µl of cDNA washing buffer + Igepal per tube, resuspend without making air bubbles
- Place in the magnet rack and wait until beads are immobilized on the tubewall
- Carefully remove and collect supernatants containing gDNA to the tubes with PAA carrier see step: DNA precipitation from the supernatant
- Resuspend beads in 20 µl cDNA washing buffer+Tween without air bubbles and transfer to the new tube
- Place in the magnet rack and wait until beads are immobilized on the tubewall
- Carefully remove and collect supernatants
- Resuspend beads in 20 µl of cDNA washing buffer+Igepal without air bubbles
- Place in the magnet rack and wait until beads are immobilized on the tubewall

Remove the supernatants carefully and collect (remove 2x, so that no residues of the washing buffer remain on the in the tube).

Quality control WTA

3 **Quality control of the WTA.docx**

3.1 **Prepare Primer-Mix:**
8 µl of each primer (stock 100 µM) = 6 Primer x 8 µl + 152 µl PCR-H_2O

All pipetting steps are performed on ice in the sterile bench.
To control the PCR reaction, a positive control (a 3-fold positive known WTA sample) and a negative control (PCR water) are included in each PCR run.

Prepare mastermix:
For the preparation of the master mix, prepare enough for the WTA samples, positive and negative controls plus an additional spare volume.

Reagents	For one sample
10x FastStart PCR Buffer (20 mM MgCl2 included)	1.0 µl
Primer-Mix (contains 3 pairs of primers)	1.0 µl
dNTPs (10 mM)	0.2 µl
BSA (20 mg/ml)	0.2 µl
FastStart Taq Polymerase (5 U/µl)	0.1 µl
PCR-Water	7.0 µl
cDNA Template	0.5 µl

- 9.5 µl of master mix are placed into a 0.2 ml reaction tubes per each sample and 0.5 µl template or positive or negative control added. Centrifuge all batches briefly and transfer to a thermocycler.
- The amplification in the thermocycler takes place according to the following program

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	Temperature	Time (h:min:sec)	Cycles
Step 1	95.0 °C	00:04:00	
Step 2	95.0 °C	00:00:30	32 cycles
Step 3	58.0 °C	00:00:30	
Step 4	72.0 °C	00:00:90	
Step 5	72.0 °C	00:07:00	
Step 6	4.0 °C	forever	

Re-amplification of WTA

4 Re-amplification of WTA.docx

4.1 Prepare mastermix:

All pipetting steps are performed on ice in the sterile bench.
For the preparation of the master mix, prepare enough for the WTA samples plus an additional spare volume.

Reagent	For one sample
Expand Long Template Buffer 1 (Buffer 1)	5.0 µl
CP2_9C (24 µM)	6.0 µl
dNTPs (10 mM)	1.75 µl
Formamide (20%)	7.5 µl
Pol Mix (5 U/µl)	1.5 µl
PCR-H2O	27.25 µl

- Place 49 µl of master mix in a 0.2 ml reaction tube per each sample
- Add 1 µl of template (cDNA from single or small number of cells)
- Briefly vortex all batches, centrifuge and transfer to a thermocycler.
- The amplification in the thermocycler takes place according to the following program:

	Temperature	Time (h:min:sec)	Cycles
Step 1	95.0 °C	00:01:00	
Step 2	94.0 °C	00:00:15	5 cycles
Step 3	55.0 °C	00:01:00	
Step 4	65.0 °C	00:03:30	
Step 5	94.0 °C	00:00:15	3 cycles
Step 6	55.0 °C	00:01:00	
Step 7	65.0 °C	00:03:30 + 10 sec/cycle	
Step 8	65.0 °C	00:07:00	
Step 9	4.0 °C	forever	

Quality control of re-amplified WTA

5 Quality control of the WTA.docx

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5.1 Prepare mastermix

For each batch, place 9 µl of master mix in a 0.2 ml reaction vessel and add 1 µl of a 1: 5 dilution of the re-amplified WTA sample or positive or negative control. Centrifuge all batches briefly and transfer to a thermocycler.

Reagents	For one sample
10x FastStart PCR Buffer (20 mM MgCl2 included)	1.0 µl
Primer-Mix (contains 3 pairs of primers)	1.0 µl
dNTPs (10 mM)	0.2 µl
BSA (20 mg/ml)	0.2 µl
FastStart Taq Polymerase (5 U/µl)	0.1 µl
PCR-Water	6.5 µl
cDNA Template (1:5 dilution of the WTA re-amp product)	1 µl

Amplification in the thermocycler is performed exactly as for primary WTAs (see above).

Endpoint PCR for target gene

6 All pipetting steps are performed on ice in the sterile bench.
For the preparation of the master mix, prepare enough for the WTA samples plus an additional spare volume. Primary or re-amplified WTA product was diluted five times with water and used as template in each test PCR (1 µl per sample)

Prepare mastermix:

Reagent	**For one sample**
10× PCR buffer	1.0 µl
Forward primer (8 µM)	0.5 µl
Reverse primer (8 µM)	0.5 µl
BSA (10 mg/ml)	0.25 µl
PANScript DNA Polymerase (5 U/µl)	0.1 µl
PCR-H2O	6.65 µl

Add figure legend here (optional)

- Place 9 µl of master mix in a 0.2 ml reaction tube per each sample
- Add 1 µl of template (five times diluted cDNA from single or small number of cells)
- Briefly vortex all batches, centrifuge and transfer to a thermocycler.
- The amplification in the thermocycler takes place according to the following program:

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	Temperature	**Time (h:min:sec)**	**Cycles**
Step 1	95.0 °C	00:04:00	
Step 2	95.0 °C	00:00:30	42 cycles
Step 3	TA	00:00:30	
Step 4	72.0 °C	00:01:30	
Step 5	72.0 °C	00:07:00	
Step 6	4.0 °C	forever	

Processing of WTA samples

7 Purification of WTA sample.docx

7.1 Preparation before the start:

- First of all, you should consider whether you want to clean up the entire material at once, or whether a part of it is sufficient. If you do not purify all the material, you have a back-up of the sample available, if an error happens. If only part of it is to be purified, transfer the desired volume to a fresh 0.2 ml tube.
- If you want to purify more than 40 µl primary/re-amplified WTA you have to transfer all samples to 1.5 ml tubes, because 0.2 ml tubes are too small for the first step.
- Add ethanol (96-100%) to the PE buffer if used for the first time. The required amount of ethanol is indicated on the bottle. Then place a check mark in the box on the lid and write the date and personal abbreviation to document that ethanol was added.
- Set the centrifuge to 17,900 x g (13,000 rpm).
- Provide and label the required number of QIAquick columns in 2 ml collection tubes.

7.2 Purification:

- Add 5 times the volume of PB buffer to each sample. Example: For purification of 20 µl per sample this would be 5x20 µl = 100 µl PB buffer (final volume: 120 µl)
- To bind the DNA, mix the samples sequentially by pipetting so that no streaks are visible
- Apply the samples to the respective QIAquick column.
- Centrifuge 60 sec at 17,900 x g.
- Discard the flow through from the collection tube and place the column back on the same collection tube.
- For washing, add 750 µL of PE buffer to each sample, centrifuge 60 sec at 17,900 x g, discard the flow through from the collection tube and place the column back on the same collection tube.
- Centrifuge the columns again to the same 60 sec collection tube at 17,900 x g to remove residual wash buffer.
- Place each QIAquick column on a clean, autoclaved 1.5 ml tube and label it.
- For elution, pipette PCR-H_2O directly onto the filter of each column without touching the filter with the pipette tip. The required volume depends on the desired concentration. In doing so, do not fall below 10 µl, as otherwise the membrane can no longer be wetted completely and losses occur. Recommended volume for eluting WTA Reamps is at least 20 µl. If the expected concentration is very high, more should be used to ensure complete elution.
- Incubate the columns for 5-10 min at RT
- Centrifuge for 30 sec at 500 x g
- Centrifuge 60 sec at 17,900 x g
- Label the tubes with eluted purified sample

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8 Concentration measurement.docx

8.1 Concentration measurement:

- Vortex the purified WTA samples briefly and centrifuge.
- Start Nanodrop2000 software and select "Nucleic Acid" program. The program should be preset to "DNA".
- Apply 1 µl of PCR-H_2O used for elution to the Nanodrop and click on "Blank". This serves to measure the background in the water. Therefore, it is important to use the same water as in the elution.
- Successively apply 1 µl of each sample to the Nanodrop and click on "Measure". After each measurement clean the arm of the Nanodrop to avoid mixing of the samples.
- Repeat the procedure for each sample

Store the purified cDNA at -20°C

8.2 Evaluation

The quality of the samples can be read on the 260/230 nm and 260/280 nm absorption ratios.
260/280 nm: primary quality feature (optimum value ~ 1.8)
Significantly lower value indicates contamination with proteins.
260/230 nm: secondary quality characteristic (optimal range 2.0-2.2)
A much lower value indicates other contaminants that absorb at 230 nm.

qPCR

9 qPCR analysis.docx

9.1 Preparation of samples:

The samples will be first diluted using PCR-H_2O at a final concentration of 1 ng/µl.

Preparation of qPCR Mastermix (MM):

Table 1 shows the compositions of the mastermix. Prepare the respective amount for your samples.
Attention: SyBr Green is light sensitive. Avoid working in the light!

Reagent	1x (15 µl)
iQTM SYBR® Green Supermix (BioRad)	10
Fwd Primer (8 µM)	1
Rev Primer (8 µM)	1
PCR-H2O	3

Pipette the qPCR plate:

1. Distribute 15 µl of the MM per well of the 96-well plate using the multipipette.
2. Add 5 µl of the diluted sample to the MM.
   As negative control, use PCR-H_2O instead of cDNA (NTC).
   As a standard, use a calibrator DNA (Cal).
   All samples should be measured in triplicates.
3. Seal the plate using a transparent foil.
4. Vortex the plate, centrifuge shortly to spin the liquids down.
5. Place the plate into the Cycler (here: LightCycler, Roche).
6. Use the following program:

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Program: **qPCR**				
	Temperature	Time (h:min:sec)	Cycles	Temp. rate
Pre-incubation	95.0°C	00:05:00	1 cycle	4.4°C/s
Amplification	95.0°C	00:00:20	38 cycles	4.4°C/s
	TA	00:00:15		
		00:00:15		
Melting curve	95.0°C	00:00:05	1 cycle	4.4°C/s
	50.0°C	00:01:00	1 cycle	2.2°C/s
	95.0°C	00:00:30	1 cycle	0.11°C/s
Cooling	4.0°C	forever	1 cycle	2.2°C/s

Analysis of generated data

Quality check and data export using Light Cycler Software

- NTC: Negative control should be negative.
- Melting curves of samples: Check whether only the desired amplicon was generated. Exclude samples showing multiple or unspecific peaks.
- Absolute quantification: Check whether amplification shows a sigmoid curve. Export Cp values and analyze them. Samples with average Cp-values >33 are considered as negative.

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