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Imaging of single cell gene expression via *in situ* RT-PCR on the Eppendorf Mastercycler® nexus flat

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Abstract

In situ RT-PCR is a useful tool to detect cell types in tissue sections. Results obtained from protein expression via immunohistochemistry or quantification of mRNA by quantitative real-time RT-PCR can be checked and correlated with this method.

In this Application Note it is demonstrated how *in situ* RT-PCR performance on the Mastercycler nexus flat contributes to high quality images of a single cell gene expression assay.

Introduction

For the detection of mRNA transcripts on the cellular level two methods are applicable: *in situ* hybridization and *in situ* RT-PCR. In certain tissues which exhibit a high content of digestive enzymes, such as in the exocrine portion of the pancreas, it is impossible to image mRNA transcripts inside the endocrine cells, or the immune cell infiltrate within the pancreatic islets, due to high background staining effects of the exocrine parenchymal cells which is induced by the different pre-treatment steps during the *in situ* hybridization procedure. Additionally, no single cells are clearly detectable in the sections developed. To overcome these limitations we modified pre-existing *in situ* RT-PCR protocols [1; 2] in order to identify endocrine cells in the pancreatic islets as well as immune cells in the infiltrate during diabetes development in pancreatic sections [3-5]. In this study, we evaluated the performance of this specific *in situ* RT-PCR protocol on the Eppendorf Mastercycler nexus flat. The results were compared directly to those obtained with a thermal cycler made by a different manufacturer, on which this method was established and which has been used in our lab for several years.
We first reduced the ramping speed on the Mastercycler nexus flat in order to achieve incubation times comparable to those of the other cycler. In subsequent experiments we selected the pre-programmed maximum ramp rate and checked for differences in signal intensities when working at this faster ramping speed which resulted in a shorter total run time.

Material and Equipment

Preparation of slides of tissue sections for *in situ* RT-PCR
Pancreatic sections of murine and human origin were used for these investigations.
2 µm thick sections cut from fixed paraffin-embedded tissue (FFPE) (4 % para-formaldehyde buffered in PBS), e.g. spleen, pancreas or kidney, were arranged on three-chamber-slides (superfrost plus, epoxy, custommade, Thermo Fisher Scientific® Schwerte, Germany). The slides containing the tissue sections were baked for 6 hours at 60 °C on a heating plate and stored in an incubator at 45 °C until further use.
The complete work process extends over two days. The de-waxing, fixation and digestion steps of the sections on the slides were performed on day 1.

De-waxing process
2 x 5 min Histo-Clear ➞ 1 min 100 % ethyl alcohol ➞ 1 min 90 % ethyl alcohol ➞ 1 min 80 % ethyl alcohol ➞ 1 min 70 % ethyl alcohol ➞ 1 min 50 % ethyl alcohol ➞ 1 min ultrapure water ➞ dry at 37 °C for 10 min.

Fixation
Thereafter the sections on the slides were fixed for 2 min at 100 °C on the heating block of a thermal cycler.

Pre-treatment with de-proteinization and digestion by DNase
In order to detect mRNA on the tissue sections, pre-treatment was required to resolve cross-linking. Additionally, an incubation step with Proteinase K (20 µg/mL in PBS) was performed for 15 min at 37 °C in a humidified chamber. Each slide contained 3 sections and 50 µl of enzyme solution, and the slides were mounted with a special 2 mm thick cover slip. The digestion step was inactivated by transferring the slides onto the heating block of a thermal cycler and incubating for 2 min at 95 °C. The sections were rinsed in PBS and in ultrapure water for 10 s each and thereafter air dried.

Overnight, DNase treatment (10 µL/section) at 37 °C in a humidified chamber was applied to remove the DNA. During this time the slides were mounted with cover slips. On the second day, the slides were rinsed once with DNase buffer, then twice with ultrapure water and subsequently air dried.

Reverse transcription (RT)
10 µL of the following RT reaction solution was pipetted to each chamber:
Nucleotide mix (dATP, dGTP, dCTP, dTTP, 1 mM each; Ares Bioscience, Cologne, Germany), 1 µM oligo-dT primer (Invitrogen®, Paisley, UK), M-MLV reverse transcriptase (0.5 U/µL) (Invitrogen®, Paisley, UK), 20 U RNasin (Promega®, Wisconsin, USA) and ddH2O. The slides were incubated at 37 °C for 1 h in a moist chamber, followed by inactivation of the reaction at 92 °C for 2 min. The cover slips were removed and the sections on the slides were washed twice with ultrapure water. The slides were stored in water until further usage.

***In situ* PCR**
15 µL of the following *in situ* PCR reaction mix were applied to each chamber:
Nucleotide mix (10 µM digoxigenin (Dig) 11-dUTP, 190 µM dTTP, 200 µM each of dATP, dCTP, dGTP; DIG-11dUTP, Roche®, Mannheim, Germany), unlabeled dNTPs (Ares Bioscience, Cologne, Germany), specific forward and reverse primers (1.25 µM), Taq BioThermT™ polymerase (0.1 U/µL) (Ares Bioscience, Cologne, Germany), selfseal reagent (MJ Research®), and ddH2O. The slides were sealed with 2 mm thick cover slips and then placed in the Mastercycler nexus flat or the other thermal cycler, respectively.

The *in situ* PCR was performed according to the following protocol on both cyclers:

95 °C	95 °C	57 °C	72 °C	72 °C	10 °C
3 min	45 s	45 s	45 s	10 min	hold
	35 – 40 cycles				

Initially the ramping speed of the Mastercycler nexus flat was decreased to achieve a total run time comparable to that of other thermal cycler. Afterwards, slides were run at the default ramping setting (max. speed) on the Mastercycler nexus flat.
Following the run, the slides were incubated in PBS for 10 min to remove the cover slips.

Blocking
Thereafter a blocking step was performed for 1 h at room temperature using 1 % blocking reagent suitable for Digdetection (Roche®), diluted in PBS.

Chromogenic detection
To detect the incorporated anti-Dig nucleotides, the slides were incubated with anti-Dig AP (alkaline phosphatase) antibody (1:500 in 1 % blocking solution) (Roche®, Mannheim, Germany) for 1 h at room temperature, followed by a rinsing step with PBS.
Alkaline phosphatase activity was detected by the NBT/BCIP (nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate toluidine salt, ready-to-use solution) (Roche®, Mannheim, Germany) color reaction and followed by a rinsing step with water. Thereafter counterstaining with hematoxylin (Zytomed, Berlin, Germany) was performed for 3 min at room temperature.
Washing steps were performed by rinsing with water, incubation for 30 s in PBS, and rinsing with water again. After mounting the slides, detection and image documentation were carried out on the BX61 microscope (Olympus®, Hamburg, Germany).

Results and Discussion

Analysis of the *in situ* RT-PCR developed sections:
In order to focus on the main differences between the two thermal cyclers, only the results obtained on the Mastercycler nexus flat at maximum speed are presented and discussed below.
Depending on the primers used, specific staining was detected either in the immune cell infiltrate or in the endocrine cells of the pancreatic islets. Results obtained from both cyclers led to the same basic conclusion. However, the results obtained with the Mastercycler nexus flat allowed for clearer and simpler interpretation based on the following reasons (Fig. 1, Fig. 2):

> The background staining of the other parenchymal cells was reduced.
> Specific staining of the mRNA transcripts was more pronounced in both the immune cells and the endocrine cells.

This improved "signal to noise ratio" may be due to the decreased temperature exposure achieved by the higher ramp rates and shortened run time. The total run time on the Mastercycler nexus flat was determined to be approx. 2 h 30 min, which was approx. 120 min shorter than the run time required when using the other thermal cycler.
The swelling process of the tissue section caused by heating during the PCR process was reduced to a larger degree in the Mastercycler nexus flat as compared to the competing thermal cycler. Therefore the images obtained from the mouse pancreas from a diabetic animal model (NOD mouse) were able to locate the mRNA transcripts of the immune cell marker FoxP3 to the small rim of a high number of T-lymphocytes (Fig. 1).

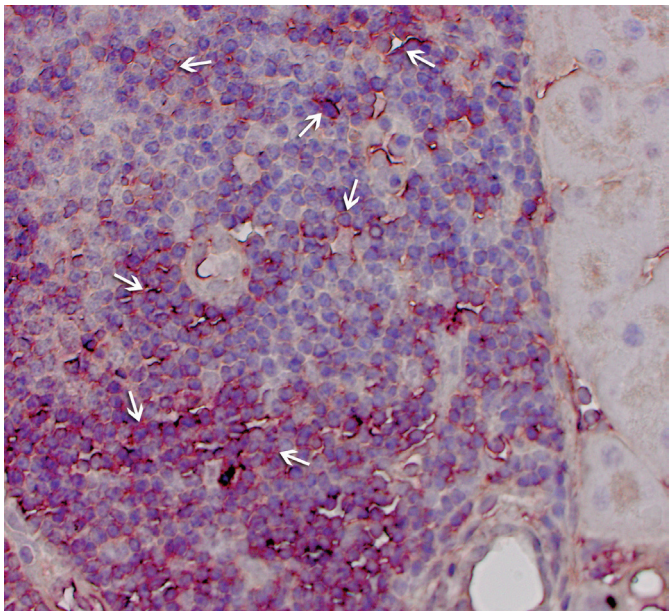

Figure 1: FoxP3 mRNA expression in the immune cell infiltrate of a pancreatic islet of a NOD mouse, detected after performing in situ RT-PCR on the Mastercycler nexus flat. A high number of immune cells expressed the FoxP3 gene (arrows) as a sign of regulatory potential. The remaining endocrine cells showed no gene expression of this marker. The surrounding exocrine parenchyma did not show high background staining from NBT/BCIP color development.

Furthermore, mRNA detection of preproinsulin enabled identification of the remaining beta cells after diabetes manifestation in the pancreatic islets (Fig. 2). This method would be functionally important if the newly synthesized proteins were not stored and immediately secreted as is the case for pro- and anti-inflammatory cytokines in activated immune cells during the process of inflammation. Therefore specific mRNA transcripts could also be detected in tissue with high expression of digestive enzymes surrounding the islets, such as the exocrine pancreatic parenchyma. The detection step showed no alterations caused by pathological changes such as immune cell infiltration into the pancreatic islets during diabetes development.

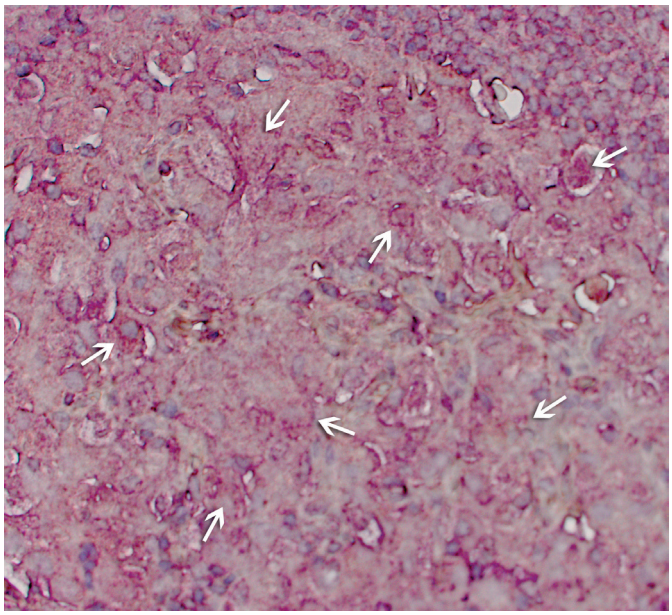

Figure 2: Insulin mRNA expression in the endocrine cells of a pancreatic islet of a NOD mouse, detected after performing in situ RT-PCR on the Mastercycler nexus flat. In the diabetic situation the remaining beta cells showed well granulated preproinsulin gene expression (arrows) within their cytoplasm. The surrounding exocrine parenchyma showed no high background staining from NBT/BCIP color development.

Conclusion

In summary, the Mastercycler nexus flat is ideally suited for this *in situ* RT-PCR method; compared to results obtained with the competing thermal cycler, the integrity of the morphology of the tissue sections was generally better preserved. Besides protein expression via immunohistochemistry or quantification of mRNA by quantitative real-time RT-PCR, the detection of cell types in tissue sections using *in situ* RT-PCR offers a useful additional tool to correlate the results obtained from the two other methods on the cellular level.

References

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Ordering information

Mastercycler nexus with silver block	International Order no.	North America Order no.
Mastercycler® nexus GSX1	6345 000.010	6345000028
Mastercycler® nexus SX1	6346 000.013	6346000021
Mastercycler® nexus GSX1e*	6347 000.017	6347000025
Mastercycler® nexus SX1e*	6348 000.010	6348000029
Mastercycler nexus with universal block		
Mastercycler® nexus gradient	6331 000.017	6331000025
Mastercycler® nexus	6333 000.014	6333000022
Mastercycler® nexus gradient eco*	6334 000.018	6332000029
Mastercycler® nexus eco*	6332 000.010	930001007
Mastercycler nexus with flat block		
Mastercycler® nexus flat	6335 000.011	6335000020
Mastercycler® nexus flat eco*	6330 000.013	6330000021
Accessories		
CAN_BUS connection cable, 50 cm	5341 612.006	950014008
CAN_BUS connection cable, 150 cm	5341 611.000	950014016
Self test dongle	6320 071.001	950030040
Temperature Verification System with 96 well sensor plate	6328 000.006	6328000006

* To run a Mastercycler® nexus with the suffix »eco« or »e«, a Mastercycler® nexus model without such a suffix is needed. Up to 2 units with the suffix »eco« or »e« can be connected to a Mastercycler® nexus without such a suffix. CAN_Bus connection cables are required to link cyclers together as a network.

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