

Electronic Supplemental Information (ESI) for Quantifying mRNA Levels Across a Histological Section with 2D-RT-qPCR

Michael Armani, Michael A. Tangrea, Benjamin Shapiro,
Michael R. Emmert-Buck, and Elisabeth Smela*

Primer Sequences Used in This Study

Table-Sup- 1. Primer sequences

Primer sets (Biosearch Technologies)

Target	Gene annotation	Forward (5' to 3')	Reverse (5' to 3')
Mouse Liver GYS2	<u>NM_145572</u>	GCCAGACACCTGACACTGA	TCCGTCGTTGGTGGTGATG
Mouse Kidney KCNJ1	<u>NM_019659</u>	GGCGGGAAGACTCTGGTTA	GTGCCAGGAACCAAACCTA
Mouse Control HPRT1	<u>NM_013556</u>	GCAAACCTTTGCTTTCCTGG	ACTTCGAGAGGTCCTTTTCACC
Target	Probe (5' to 3')		
Mouse Liver GYS2	5'CalFluorOrange560-TTTCAGACAAATTCACCTAGAGCCC-BHQ1		
Mouse Kidney KCNJ1	5-FAM-AAGCACCGTGGCTGATCTTCCAGA-BHQ1		
Mouse Control HPRT1	5'Quasar670-CAGCCCCAAAATGGTTAAGGTTGCAAG-BHQ2		

The GYS2 and KCNJ1 primer and probe sets target both DNA and RNA with amplicon sizes of 73 and 67 bases. The HPRT1 target is intron spanning and only amplifies RNA efficiently with an amplicon size of 85 bases.

Specific Protocol for Duplex mRNA Mapping (ChargeSwitch Buffer)

The workflow outlined in section 3.2 of the manuscript was followed with a few modifications. First, a tissue section containing mouse kidney, mouse brain, and chicken thymus was sectioned on adhesive film and treated with eosin Y stain as described. This tissue on adhesive film was then thawed at 90 °C for 15 minutes, and transferred onto part of a 384-well plate – a 6x5 grid of wells – pre-loaded with ChargeSwitch lysis buffer. The film was sealed for 2 minutes total time. The fluid was distributed over the tissue by manually inverting the plate 5 times (instead of inverting the plate once). After lysis, ChargeSwitch beads and binding buffer were added to each well (0.33 µL ChargeSwitch magnetic beads, 0.4 µL acetic acid, 5.75 µL ChargeSwitch binding buffer, and 8.52 µL water). A wash was performed using 12.5 µL of wash #14, 6.25 µL of DNase was added and incubated for 10 minutes, and 2 µL of ChargeSwitch binding buffer (B9) was added. After removing the DNase buffers, two washes were performed using 20 µL of wash #13 and 12.5 µL of wash #14 buffers.

AgPath-ID one-step RT-PCR mix was prepared according to the manufacturer's protocol. The mix included final concentrations of 250 nM primers and 125 nM probes for both HPRT1 and KCNJ1

targets. Five μL of the PCR mix was added to each well in the 6x5 grid of wells. The thermocycling parameters were 50 °C for 10 minutes, 95 °C for 10 minutes, and 30 total cycles of 95 °C for 15 seconds and 60 °C for 30 seconds. The plate was removed and imaged on a Typhoon 1410 flatbed scanner every 5 cycles from cycles 15 to 30.

Specific Protocol for Triplex mRNA Mapping (ChargeSwitch or Custom GITC Buffer)

The workflow outlined in section section 3.2 of the manuscript was followed with a few modifications. Two 5x5 grids within a 384-well plate were pre-loaded with lysis buffer. In one grid, each well was preloaded with a mixture containing 12.5 μL ChargeSwitch lysis buffer, 6.25 μL binding buffer, 0.5 μL beads, 0.2 mg/mL proteinase K, 5 mM DTT, and acetic acid to a final concentration of 2% by volume. In the second grid, each well was preloaded with 5 μL of custom GITC-based lysis buffer. Two serial recuts of tissue containing mouse liver, kidney, and heart were made as described, stained red using acid fuchsin red, and placed on the two grids. The plate was inverted, incubated, centrifuged, and washed as described. AgPath-ID one-step RT-PCR mix was prepared according to the manufacturer's protocol, with final concentrations of 250 nM primers and 125 nM probes for each of the three targets, HPRT1, GYS2, and KCNJ1. The plate was removed and imaged at cycles 10, 25, 28, 31, 34, 37, and 40 using a Tecan 200 plate reader.

Example of 2D-RT-PCR Without DNase Treatment

A workflow similar to the one outlined in section 4.5 of the manuscript was followed for triplex mRNA mapping, but with the DNase step omitted. Again, as in Figure 4 of the manuscript, all the wells that had tissue showed a signal (Figure Sup- 1) from the HPRT1 housekeeper expressed in all mouse tissues. However, in addition, fluorescent data in all the wells with tissue also showed signals for KCNJ1 and GYS2 targets, which were not intron-spanning and could therefore detect genomic DNA. Comparing this detection map with the ones in Figures 3 and 4 of the manuscript showing KCNJ1 signals only in kidney tissue, it is apparent that DNase treatment is sufficient and necessary to provide a detection map specific to mRNA.

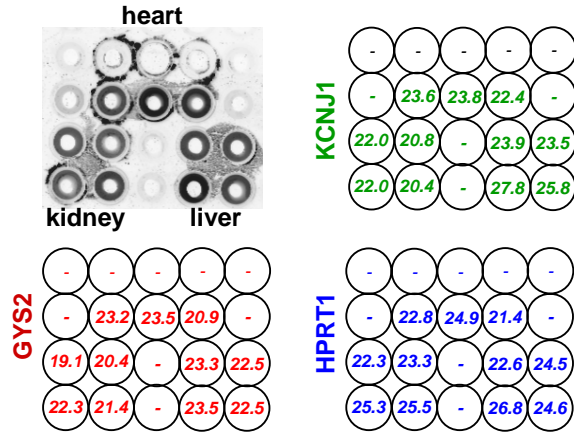


Figure Sup- 1. Fluorescent image showing the position of a mouse tissue section containing heart, liver, and kidney, and tissue lysate within wells. The fluorescence is provided by eosin Y staining of the tissue. Dark color within wells indicates that tissue was present and lysed. Three detection maps were obtained using 2D-RT-qPCR without DNase treatment. The results show the detection of GYS2, KCNJ1, and HPRT1 in all of the wells that contained tissue.

Fluorescent Data and Determination of Cts

It was necessary to perform PCR in a 384-well thermocycler, but a fluorescent plate reader was used to collect multiplex fluorescent data. Initially, a typhoon 1410 flatbed scanner was used to collect data at PCR cycles 15, 20, 25, and 30. However, a Tecan 200 plate reader was identified as a faster imaging system and was later used to measure fluorescence values. When using the Tecan to measure fluorescence for the KCNJ1, GYS2, and HPRT targets, excitation/emission wavelengths of 495/532, 538/573, and 649/679 nm were used. Because the Tecan instrument was faster, fluorescence signal data could be obtained more frequently. Data was obtained at PCR cycles 10, 25, 28, 31, 34, 37, and 40.

In order to determine Cts, the difference between two cycles was calculated as $D_i = \log(\text{data}_{\text{cycle}(i)} / \text{data}_{\text{cycle}(i-1)})$, based on the standard curve formula of PCR (38). The cycle C_i was determined when D_i was greater than a threshold value; thresholds were defined as 0.04 for KCNJ1, 0.06 for GYS2, and 0.08 for HPRT1 to filter out background fluorescence. Finally, the Ct value was calculated as $C_i - A * D_i / 0.2$ for KCNJ1 and GYS2 or $C_i - A * D_i / 0.25$ for HPRT1. The constant value A was defined as the number of PCR cycles between fluorescent images (3 or 5). Based on negative controls and background values, Cts above 30.0, 32.5, and 35.0 were ignored for KCNJ1, HPRT1, and GYS2, respectively.

Binding of DNA to Adhesives Film

An experiment was performed to determine if nucleic acids could be adsorbed onto the adhesive films used in 2D-RT-qPCR due to the application of heat. It was assumed that DNA would bind in a similar way to RNA. Thus, 5000 ng of FAM (fluorescent) labeled DNA oligonucleotides were

added to custom lysis buffer, which was added into three wells of a 384-well plate. The plate was inverted to bring the buffer into contact with the adhesive film and 60 °C heat was applied for 15 minutes. Using a second sample, the plate was inverted but no heat was applied. The two plates were centrifuged to pull the buffer back into the wells. The adhesive films were dried and then imaged using a fluorescent scanner. Using a dilution series of known amounts of dried labeled oligo as a comparison, it was determined that 2% or less of the DNA was bound to the adhesive films as a result of heating. Non-specific adsorption of DNA to surfaces is a known phenomenon and a major concern. However, the application of strongly denaturing buffer used in these experiments is expected to prevent most of the DNA from binding to the film, as was demonstrated in this experiment.