KimanTech Technical Application:
2-Step qPCR using the Alluvia System

Background

The Alluvia system is intended to enable 2-step qPCR and subsequent loading on an electrophoresis gel while keeping the PCR products continuously contained. 2-step PCR is when PCR products (typically purified or just diluted) become templates for a second round of PCR. More often, the primers for the second PCR round are positioned internally ("nested") relative to the primers used in the first round in order to improve PCR specificity because it is unlikely that the second set of primers will amplify the same unwanted region as the first set of primers. Nesting is particularly useful when the first round of PCR is prone to generating unwanted products, for example, multiplex PCR reactions.

A major problem with 2-step PCR is the elevated risk of PCR product contamination of the laboratory environment due to processing PCR products, as minute amounts of PCR products can create false results in subsequent first round PCR reactions of the same type. This risk can be reduced but not eliminated by incorporation of dUTP in the PCR reaction and subsequent selection against uracil containing templates using Uracil-DNA-Glycosylase. However, this selection method is useful for only one of the two rounds of PCR. This can be of significant concern if the lab is also exposed to the second round PCR products, particularly if they are loaded on an electrophoresis gel.

Alluvia Design and Operation

The Alluvia system is comprised of two types of consumables, the Plate Loading Manifold (PLM) and the Gel Loading Manifold (GLM), and an instrument that controls operation of the PLM. The current PLM format will accept a single standard PCR tube containing first round PCR products, dilute the products approximately 1:100, then aliquot into a standard 8-well PCR tube strip that can be subsequently thermocycled on a standard qPCR instrument. The post second round PCR products contained in the tube strip can then be injected in a sealed manner into a cartridge-type (Invitrogen E-Gel™, Lonza FlashGel™) disposable electrophoresis gel.

The system works as follows:

- The secondary PCR reactants (primers and master mix of the user's choice) are added an 8-tube PCR strip, which is then attached to the PLM. The PLM is then placed on the Alluvia instrument.
- User supplied buffer is injected into the PLM loading port filling an expandable bladder.
- The PCR tube containing first round PCR products is inserted into the PLM loading port, thereby sealing the port, rupturing the bottom of the tube, and diluting the PCR products.
- A portion of the diluted PCR products are pushed into an expandable channel located in the tube cap segment of the PLM.
- Heating elements weld this channel forming small aliquots of fluid, one aliquot located above each tube of the PCR tube strip.
- A membrane below each aliquot is ruptured allowing the liquid to flow into the associated tube in the tube strip.
- The cap portion of the PLM is cleaved from the remainder of the PLM degrading the PCR products at the cleavage site to prevent PCR product contamination.
- The tube strip with PLM cap attached is briefly centrifuged to concentrate liquid in the bottom of the tubes, then placed in a PCR/qPCR machine for the secondary reaction.

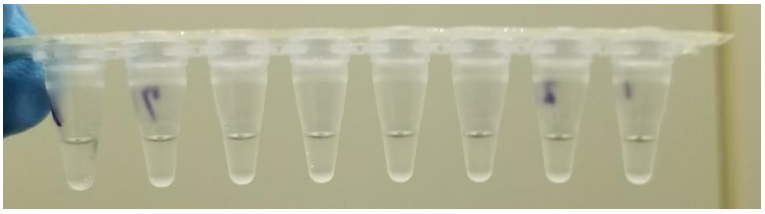
- If gel electrophoresis of the secondary PCR products is desired, the GLM can be utilized. Two gasket release liners located on the underside of the GLM are removed, then the GLM is positioned on top of the disposable cartridge gel.
- Following PCR, tube strips from the PLM are placed in the receiving slots of the GLM, then the Tube Injector positioned above the tube strip.
- Downward force is applied to the Injector, which forces the tubes into the receiving slots, then rupturing the bottom of the tubes on a razor blade located at the bottom of the slots, and injecting the contents into the electrophoresis wells.
- The Tube Injector is removed, and the gel is electrophoresed as normal.
- Following electrophoresis, the gel along with the GLM are disposed of, thereby keeping the PCR products contained and eliminating the PCR product contamination risk.

Alluvia Performance

Volume Delivery and Dilution Efficiency:

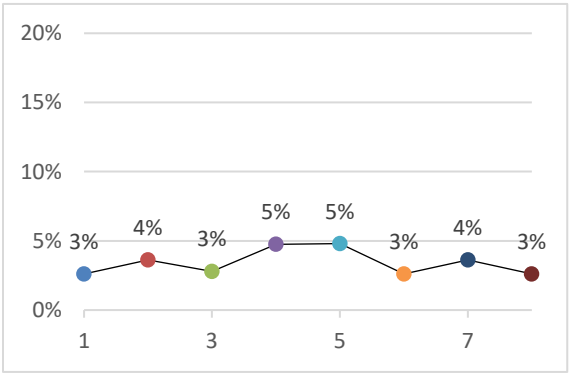
- A 10ul aliquot of 200 ng/ul salmon sperm DNA in Tris/EDTA is placed in a PCR tube, then inserted into the PLM prefilled with 1 ml TrisEDTA + 0.5% Tween-20.
- The wells of the secondary PCR tube strip (prefilled with 12 ul 1X TrisEDTA - to mimic the master mix and primers) are filled using the PLM as described above. Images of these tubes are shown in Fig. 1.

Fig 1: Example of an 8-tube strip following dilution and fluid transfer of liquid from a primary PCR tube using the Alluvia Plate Loading Manifold (PLM).



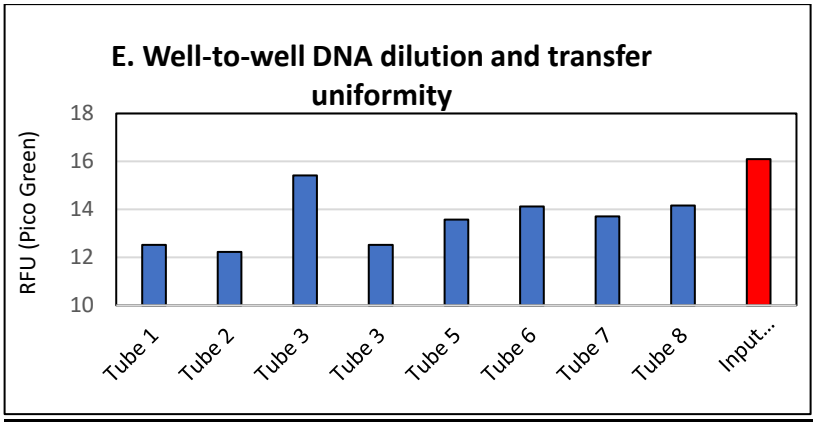
- The PLM caps are removed, and the volumes determined using the difference in weight between full and empty tubes (Fig. 2)

Fig 2: Variation in volume (CV) recovered from tubes



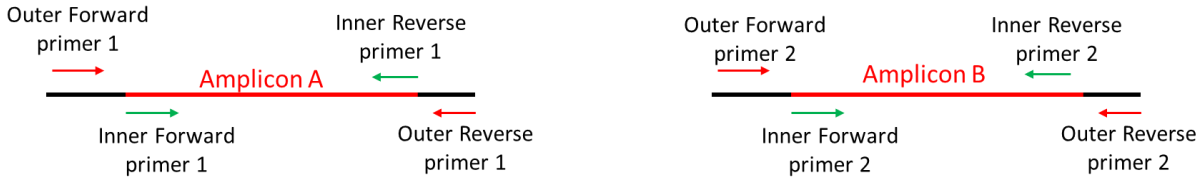
- The DNA concentration in both the input and output samples was determined using Pico Green (Fig. 3).

Fig 3: DNA concentrations in input and output samples



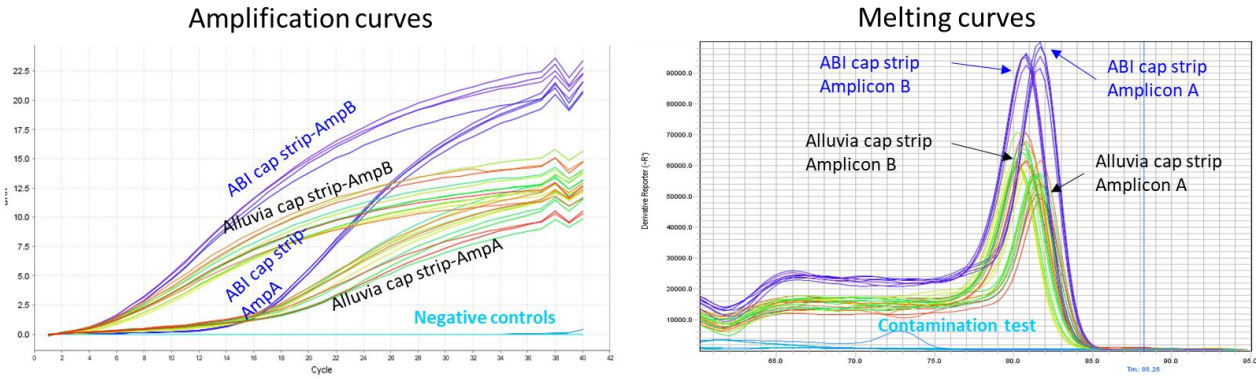
qPCR Performance and Contamination Testing:

Fig 4: PCR amplicons used in the following study



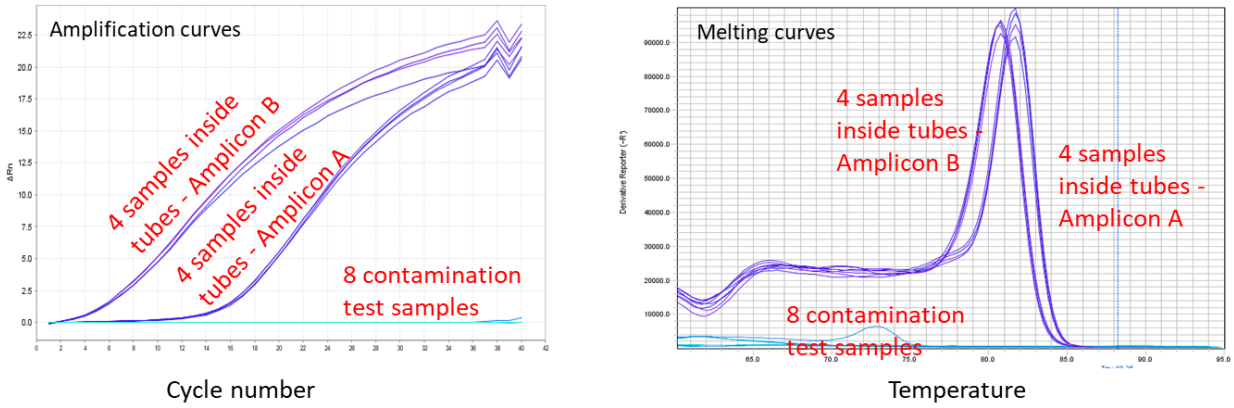
- A primary qPCR multiplex reaction using the outer primers in Fig. 4 was performed. (All PCR reactions were performed using Applied Biosystems™ (ABI) Power SYBR™ Green PCR master mix).
- Two secondary PCR reaction mixtures were set up. Amplicon A mix was aliquoted into the odd wells and amplicon B mix was aliquoted into the even wells of an 8-well tube strips, which was then attached to a PLM.
- The post PCR tubes containing the primary multiplex products were inserted into the PLM and processed as described above.
- Prior to performing the qPCR, 25 ul drops of dilution buffer were placed at eight locations on the external portion of the PLM, then 12 ul recovered and placed in either amplicon A or B mix and used for contamination control (Fig. 5)
- qPCR including melting analysis was performed using the ABI StepOne™ instrument using standard PCR conditions. Comparisons to standard ABI strips caps are shown in Fig. 5.

Fig 5: qPCR comparison using the Alluvia PLM caps vs. standard PCR tube



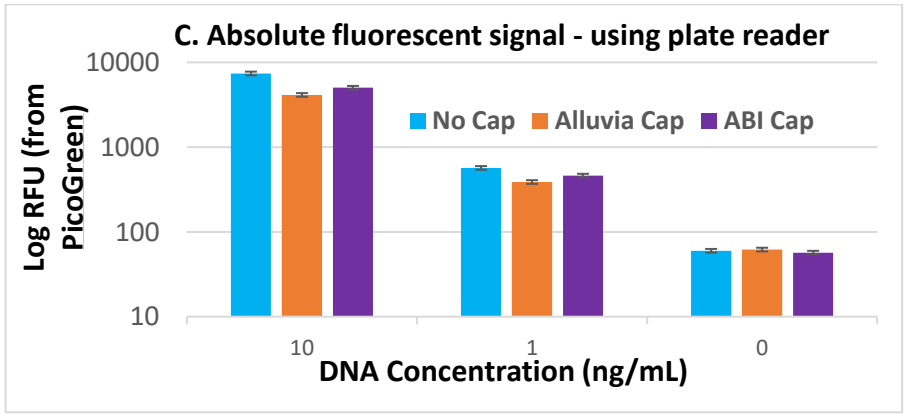
Contamination test:

8 DNA samples vs. 8 samples from liquid pipetted, then collected from 8 points on outside of post processed consumable



- Salmon sperm DNA (in Tris/EDTA) in the concentrations shown in Fig. 6 were mixed with 1:200 PicoGreen and placed various wells of a 96-well PCR plate.
- These wells were sealed with the pre-ruptured cap portion of a PLM or an ABI strip cap. Total fluorescence signal was determined using a fluorescence plate reader.

Fig 6: Absolute fluorescent signal from Alluvia PLM caps vs. standard PCR caps



Gel Electrophoresis and Contamination Testing:

- PCR reactions using the outer primers of Amplicons A and B (Fig. 4) were performed in 8-well strips (Amplicon A in odd wells, Amplicon B in even wells) using ruptured Alluvia caps.
- Using the GLM system, these post PCR strips were loaded into a 2% agarose Invitrogen E-Gel™ or a 2% agarose Lonza FlashGel™ as described above.

Fig 7: Gels loaded with the Alluvia GLM system:

A. Invitrogen E-Gel™, B. Lonza FlashGel™