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 defined as performing two rounds of PCR where the products of the first round of PCR (typically purified or just diluted) become the template for the second round of PCR. More often, the primers for the second round are positioned internally ("nested") relative to the primers used in the first round to improve PCR specificity. Specificity is improved 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 reaction.

A major issue with 2-step PCR is the elevated risk of PCR product contamination due to processing the first round PCR products. This risk can be reduced but not eliminated by incorporation of uracil and subsequent selection against uracil containing templates using Uracil-DNA-Glycosylase in subsequent PCR reactions. However, this selection method is useful in 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 and an instrument. The consumables are the Plate Loading Manifold (PLM) and the Gel Loading Manifold (GLM). The Alluvia instrument controls operation of the PLM. The current PLM format will accept a single standard 100 ul 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 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 tube cap portion of the PLM.
- The PCR tube containing first round PCR products is inserted into the PLM.
- The Interface Cap is pressed over the PCR tube. A hollow needle (imbedded in the Interface Cap) pierces the lid then the bottom of the tube, then is withdrawn slightly so the needle tip remains within the tube.
- User supplied buffer is injected into the Interface Cap. The buffer flows through the primary PCR tube collecting and diluting the primary PCR products, then flowing them into an expandable bladder in the PLM.
- A small portion of the diluted PCR products in the bladder is pushed into an expandable channel located in the tube cap portion of the PLM.
- Heating elements in the Alluvia instrument weld this channel forming small aliquots of fluid, one aliquot located above each tube of the PCR tube strip.
- The Alluvia instrument ruptures membranes in the PLM, allowing each liquid aliquot to flow into the associated tube in the tube strip.

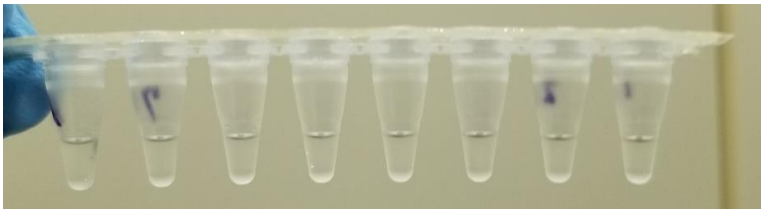
- The cap portion of the PLM is cleaved from the remainder of the PLM in the presence of bleach to prevent PCR products from being released.
- The tube strip with PLM cap attached is briefly centrifuged to concentrate liquid in the bottom of the tubes, then placed in a PCR/qPCR machine for the secondary reaction.
- If gel electrophoresis of the secondary PCR products is desired, the GLM can be utilized. Using the GLM two gasket release liners located on the underside of the GLM are removed, then the GLM is positioned on top of a commercial sealed disposable gel (i.e. the Invitrogen E-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 downward in the receiving slots, slightly compressing the tubes of the tube strip, then subsequently rupturing the bottom of the tubes on a razor blade, located at the bottom of the receiving holes of the GLM.
- The pressure generated inside the tubes ejects the tube contents into the wells of the electrophoresis gel. 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:

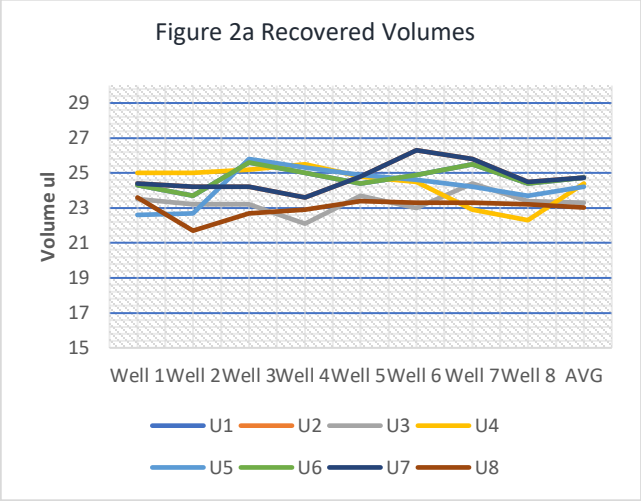
- A 10ul aliquot of 200 ng/ul salmon sperm DNA in Tris/EDTA is placed in a PCR tube, then inserted into the PLM.
- The Interface Cap was used to pierce the tube as described above, then 1 ml of TrisEDTA + 0.5% Tween-20 buffer injected as described above, thereby filling the bladder of the PLM.
- The 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.

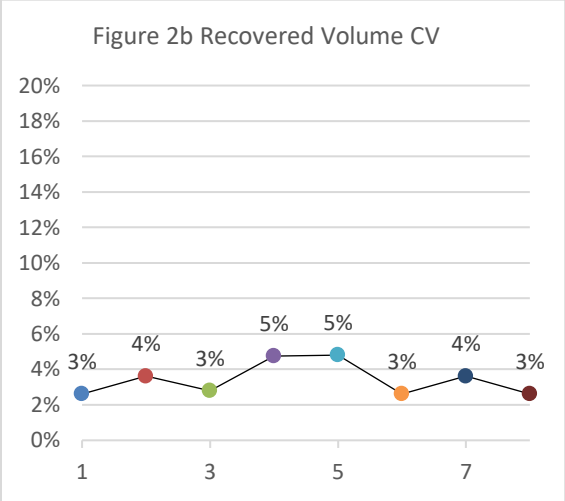
Fig 1: Example of an 8-tube strip following dilution and fluid transfer of liquid from a primary PCR tube using the Alluvia Plate Loading Manifold (PLM).



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

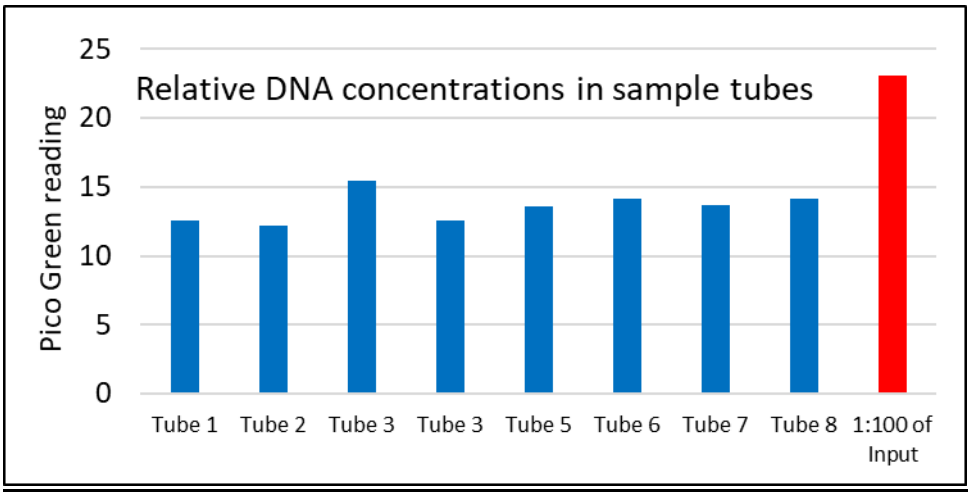
Fig 2: Recovered volumes from tubes





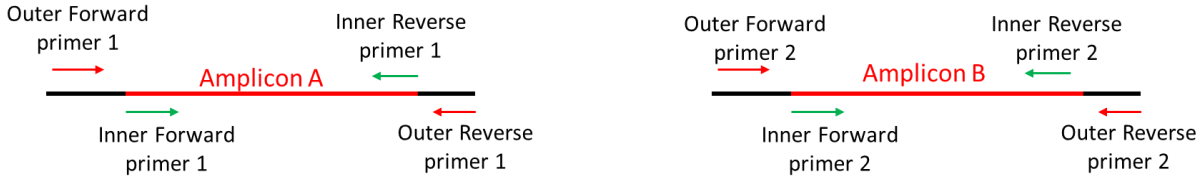
- TrisEDTA is added to bring the total volume to 100 ul. The DNA concentration in both the input and output samples was determined using Pico Green (Fig. 3).

Fig 3: DNA concentrations in input and output samples



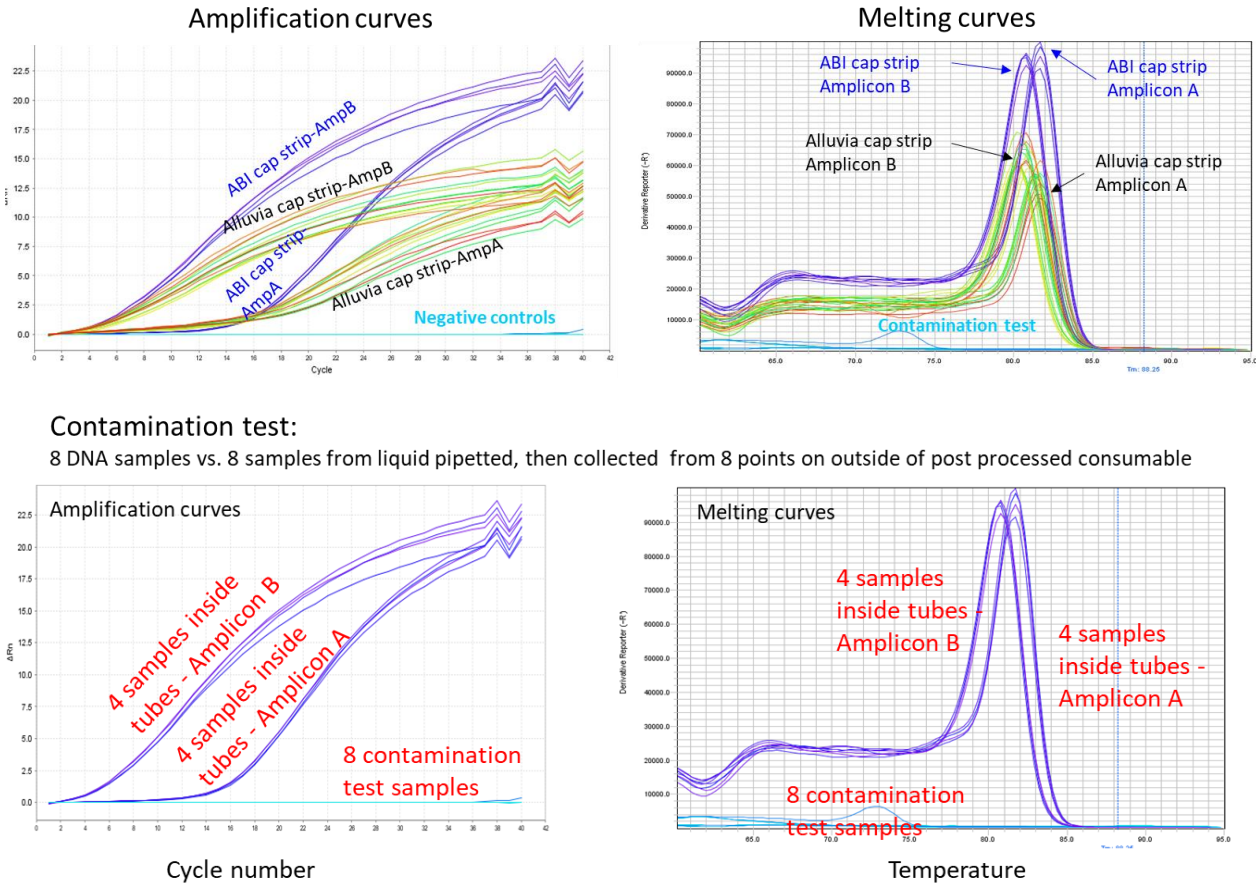
gPCR Performance and Contamination Testing:

Fig 4: PCR amplicons used in the following study



- A primary qPCR multiplex reaction (2 amplicons, using the outer primers in Fig. 4) was performed in each of two PCR tubes. The qPCR amplification and melting curves demonstrated these reactions were equivalent.
- Two secondary PCR reaction mixtures were set up by placing SYBR Power master mix (Life Tech) into each of two tubes, then placing the nested primer pair for each amplicon in one of these tubes, one pair per tube.
- Amplicon A tube mix was aliquoted into the odd wells and amplicon B mix was aliquoted into the even wells of 8-well tube strips. A tube strip 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 (using Tris/EDTA+0.5% Tween-20), thereby delivering diluted aliquots to the secondary PCR tube strips.
- Prior to performing the qPCR, 25 ul drops of dilution buffer were placed at the indicated locations on the PLMs, then 12 ul recovered and placed in either amplicon A or B mix and used for contamination control (Fig. 7)
- A portion of the remaining contents of the PLM bladder was carefully recovered and 12 ul/well was pipetted into another tube strip, then sealed using a standard (ABI) PCR cap strip.
- qPCR including melting analysis was performed using the ABI/ThermoFisher StepOne instrument using standard PCR conditions. The results are shown in Fig. 5

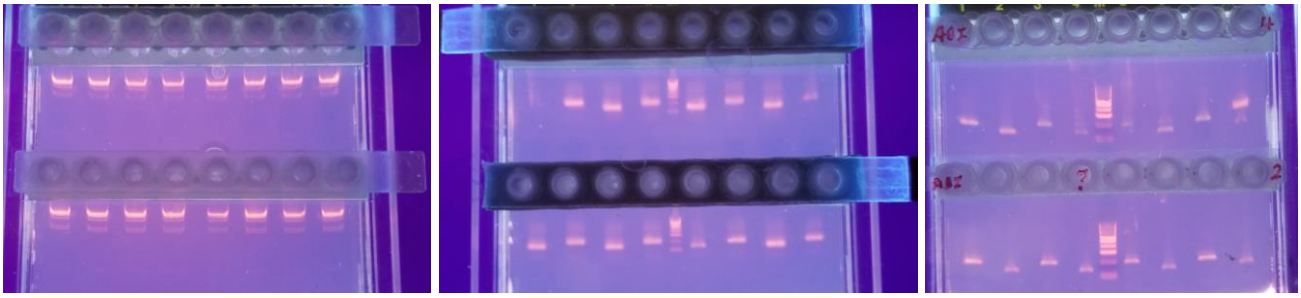
Fig 5: qPCR comparison using the Alluvia PLM caps vs. standard PCR tube



Gel Electrophoresis and Contamination Testing:

- An Alluvia PLM test strip from above was loaded into a 2% E-gel (Invitrogen/Thermo-Fisher) as described above.

Fig 6: Gels loaded with the Alluvia GLM system:
A. Lambda Hind3 size standards pipetted into tubes and Alluvia system performed without fluids,
B. PCR products generated using the Alluvia system,
C. PCR products generated using standard (ABI) cap strips.



A. B. C.