Plant Biotechnology

Bayer Russia Plant Biotechnology Conference

July 2023

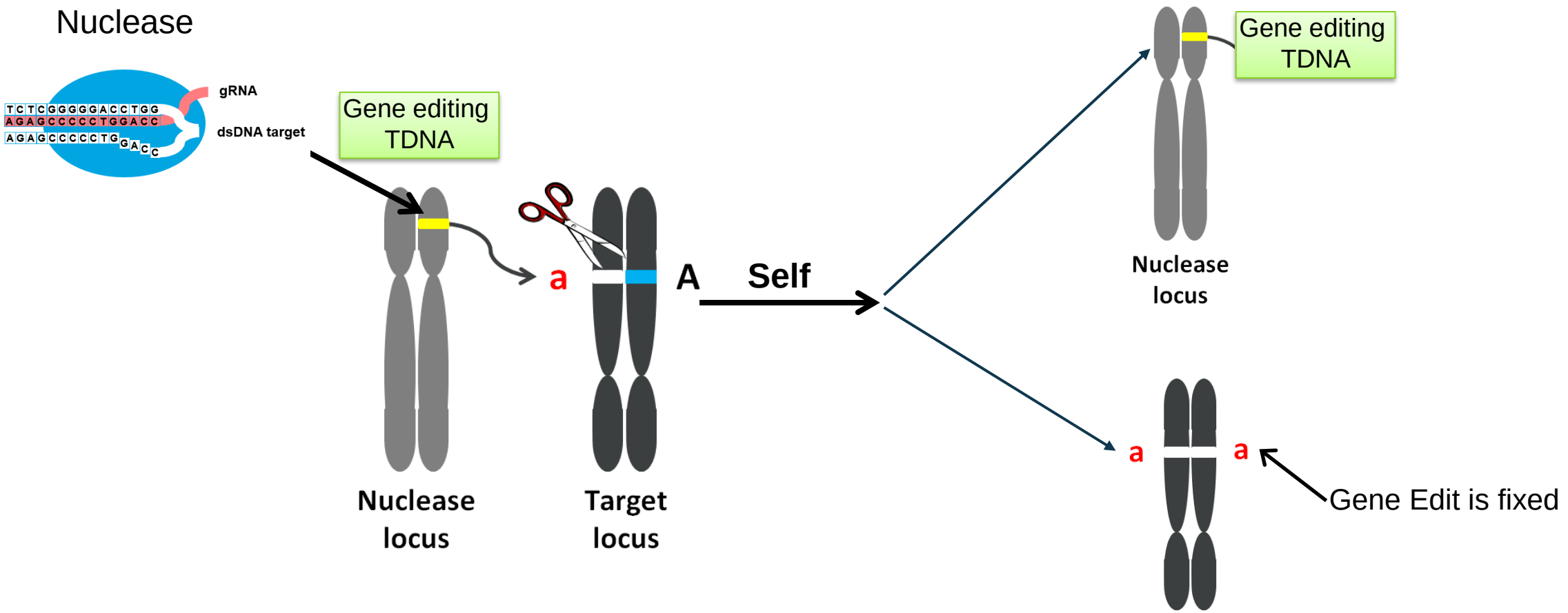
Molecular Assays

Amplicon Sequencing

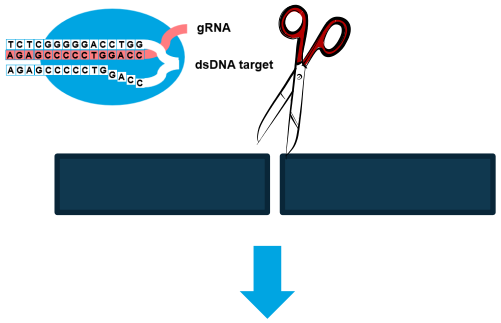
About me: Dr. James H. Crowley, PhD

- PhD in Microbiology from North Carolina State University
- Over 25 years experience in Plant Biotechnology at Bayer Crop Science
- Project lead experience leading commercial development of Biotech crops
- Eight years of experience in developing and running molecular screening assays for Biotech crops
- Four years of experience as lead of the STL TaqMan Lab in Chesterfield, MO site

Gene editing machinery enables making precise edits in the gene of interest followed by segregation of transgenic nuclease from edits



Gene edits can be advanced based on amplicon sequencing



GAGGGC__GCCATCA

GAGGGCATGCCATCA

Select for edit at T0

GAGGGC__GCCATCA

GAGGGC__GCCATCA

GAGGGC__GCCATCA

GAGGGCATGCCATCA

GAGGGCATGCCATCA

GAGGGCATGCCATCA

Select for homozygous edit at T1

GAGGGC__GCCATCA

GAGGGC__GCCATCA

Confirm homozygous for edit at T2

Use amplicon sequencing to detect and advance edits

Gene editing involves selection for edits and against TDNA at the same time in plant advancement

Self-pollinate **Self-pollinate**

TDNA
EDIT

- **Select single copy inserts**
- **Select edits**

- **Select null for TDNA**
- **Select homozygous for edits**

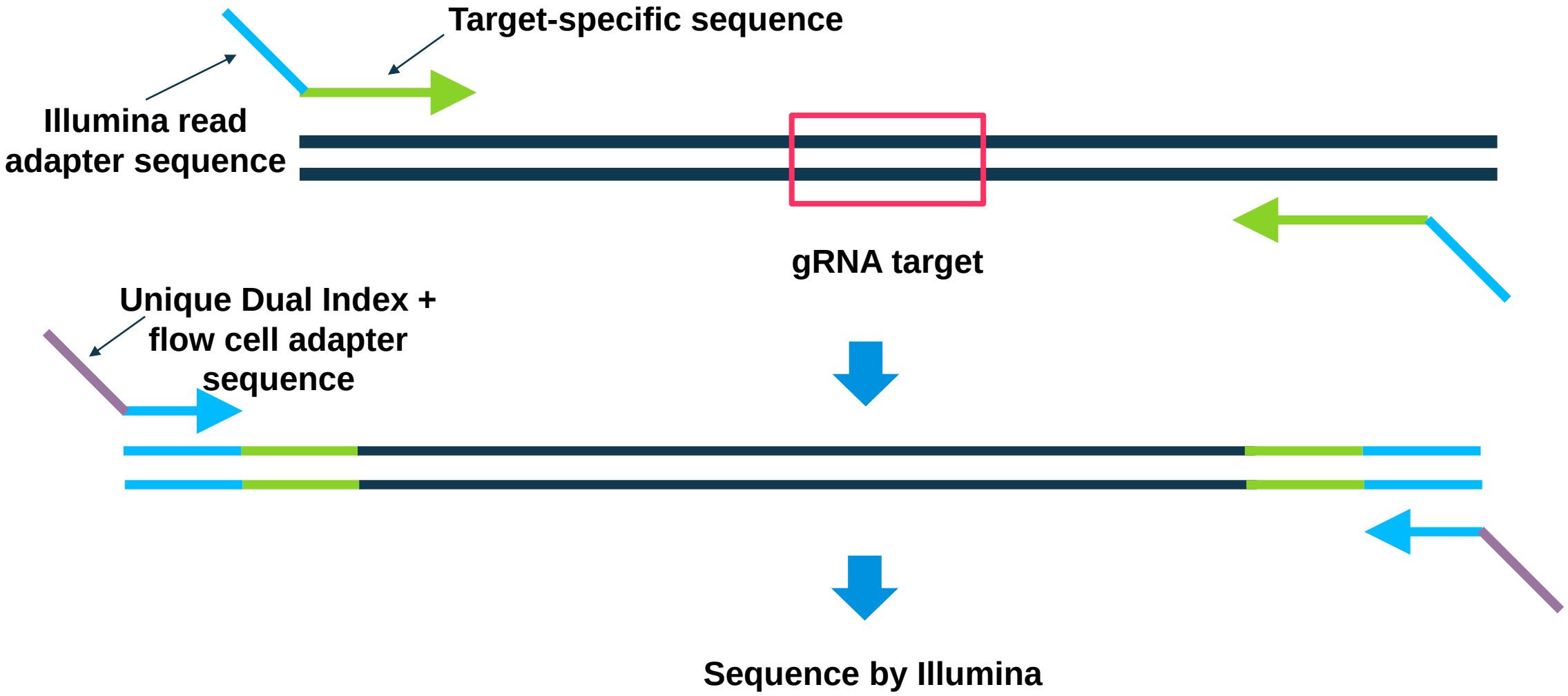
- **Confirm null for TDNA**
- **Confirm homozygous for edits**

T2 homozygous (“fixed”) lines used as parent donors for further breeding or for use in phenotypic trials to assess trait efficacy

Considerations for gene editing detection

- Sequencing provides precise detail on the type of edit and the diversity of edits at the target site within the plant
  - Sequencing allows detection of biallelic variants in different generations (where different mutations are found in each of the two different alleles)
- Amplicon sequencing of T0 provides quantitative data on edit sequence prevalence to support prediction of inheritance of the edit to next generation
  - Chimerism at T0 is to be expected where sequencing may show multiple different edits and they may be in different cells.
- Once the edit is fixed in later generations with a known sequence, a different assay may be developed (e.g. TaqMan®)
- There are other methods for gene editing detection, however, amplicon sequencing is a very precise and informative method that is the focus of this presentation
  - See Further Reading later in this presentation for references to other methods

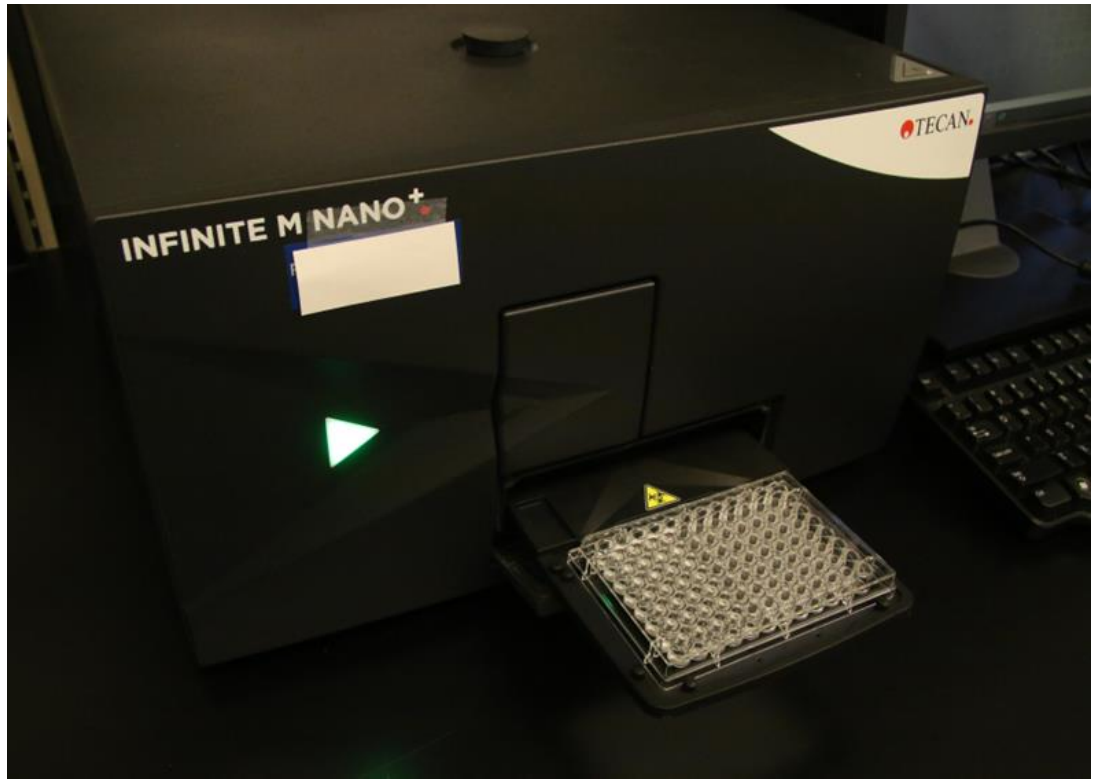
Amplicon sequencing involves a two-step PCR method



Considerations for amplicon sequencing

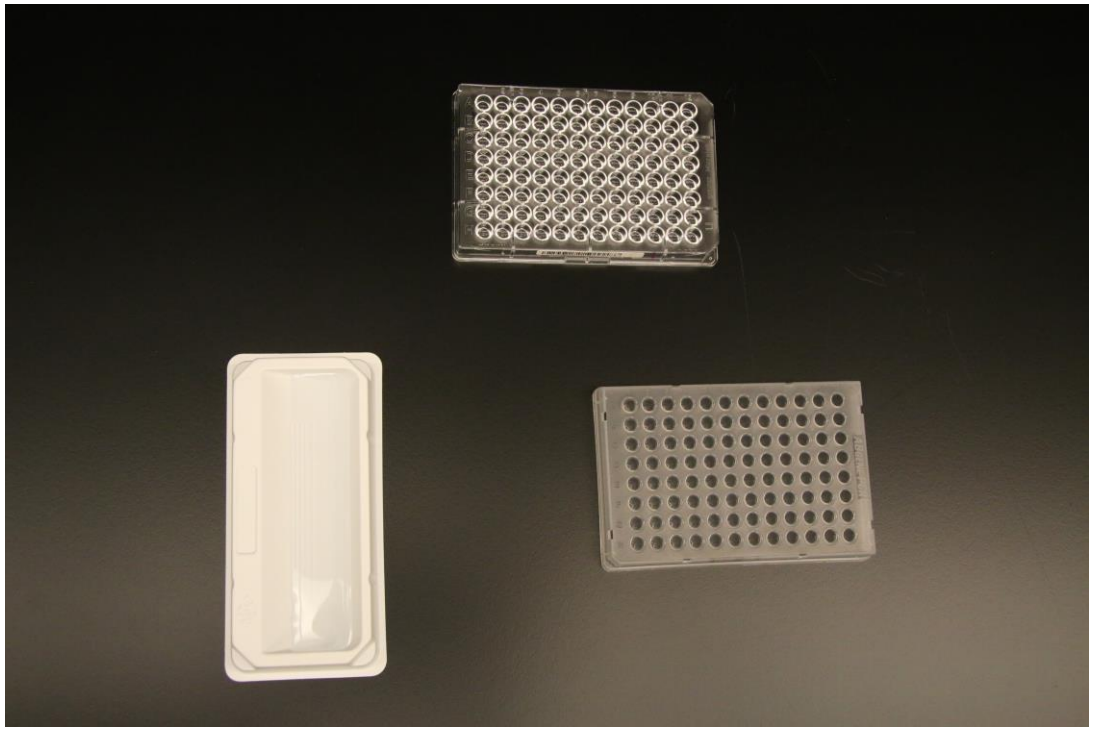
- Genomic DNA may be checked following extraction to ensure high molecular weight (large fragment) DNA is present to ensure DNA is not sheared. Agilent chip reader can be used for this
- Bead cleanup methods can be used on source DNA to remove inhibitors that may affect PCR amplification based on the extraction method and crop.
  - Bead cleanups can be used at any point in the process
- Formamide and DMSO can also be added to PCR mastermixes to help with amplification prior to sequencing
- Agarose gels, acrylamide gels, E-gels or other types of PCR amplicon visualization methods can also be used for amplicon quality control as replacements for use of Agilent bioanalyzers

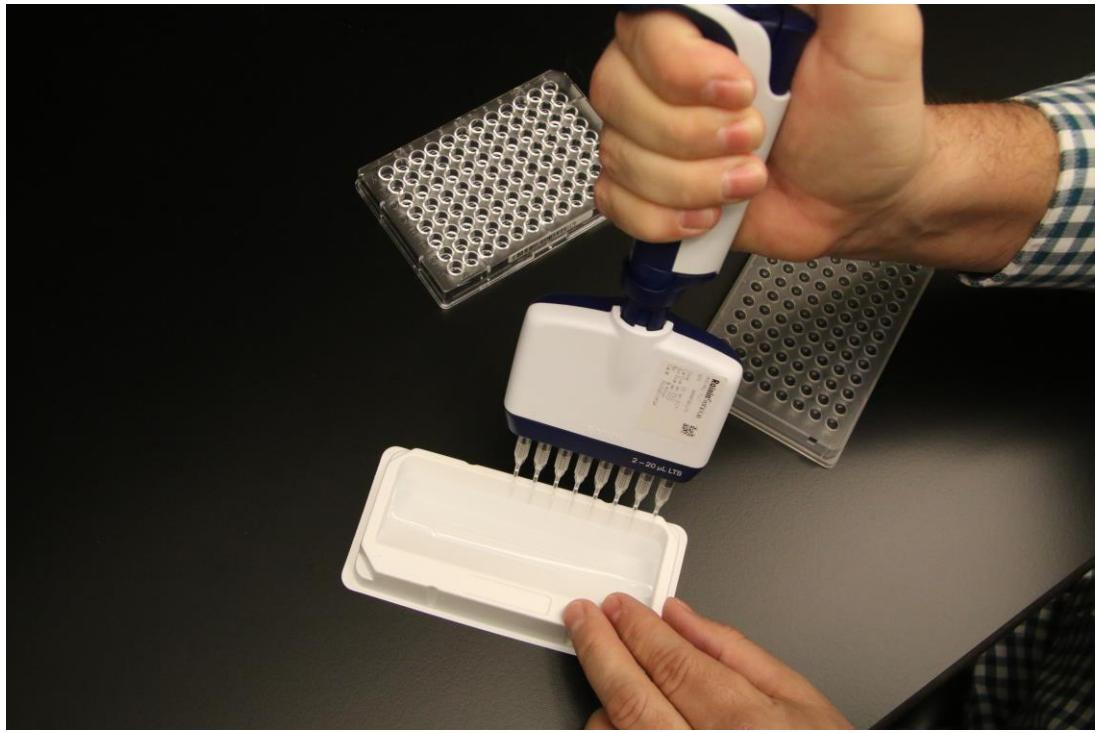
Verify Concentrations of genomic DNA using PicoGreen



- Use DNA extractions from the DNA extraction protocol, or similar
- Ensure all samples have a DNA concentration of 1-5 ng/µl
- For samples greater than 5 ng/µl, dilute to 5 ng/µl

Prepare PCR1 mastermix



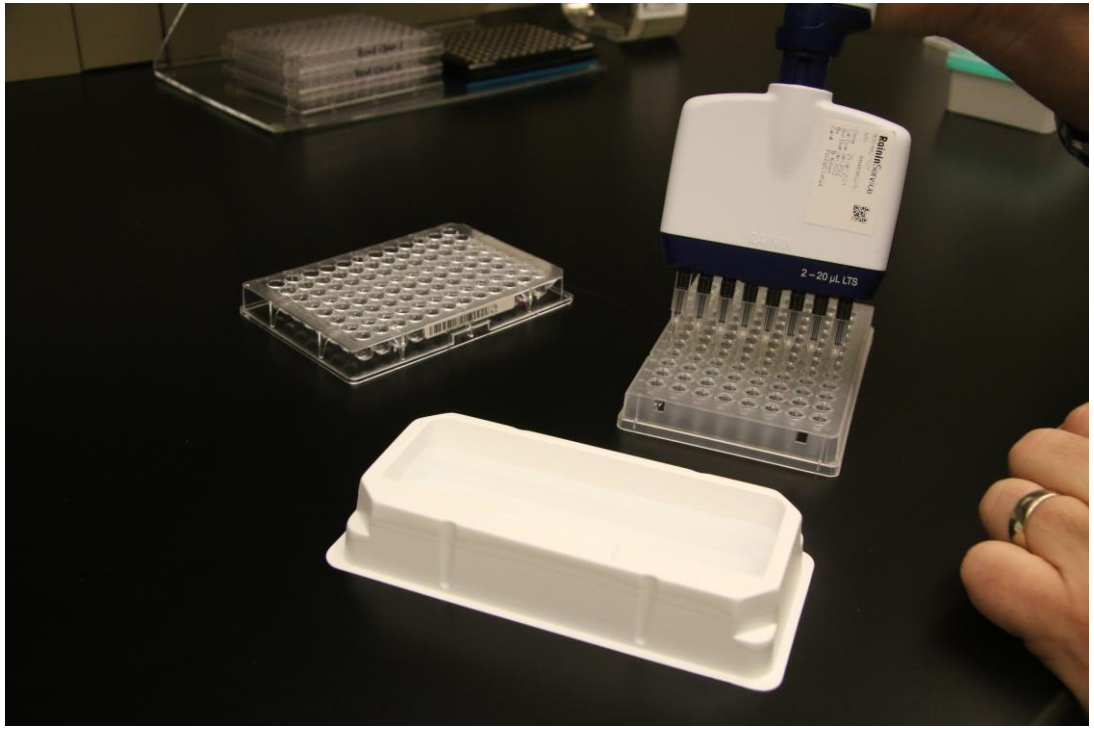


Reagent	Concentration	KAPA	Phusion
KAPA or Phusion mastermix	2X	25ul	10ul
Forward Primer	5uM	1ul	1ul
Reverse Primer	5uM	1ul	1ul
UltraPure Water		20ul	5ul

Volumes are per sample

- Assemble genomic DNA source plate, 96-well PCR plate, and reagent trough
- Mix PCR reagents

Complete setup for PCR 1

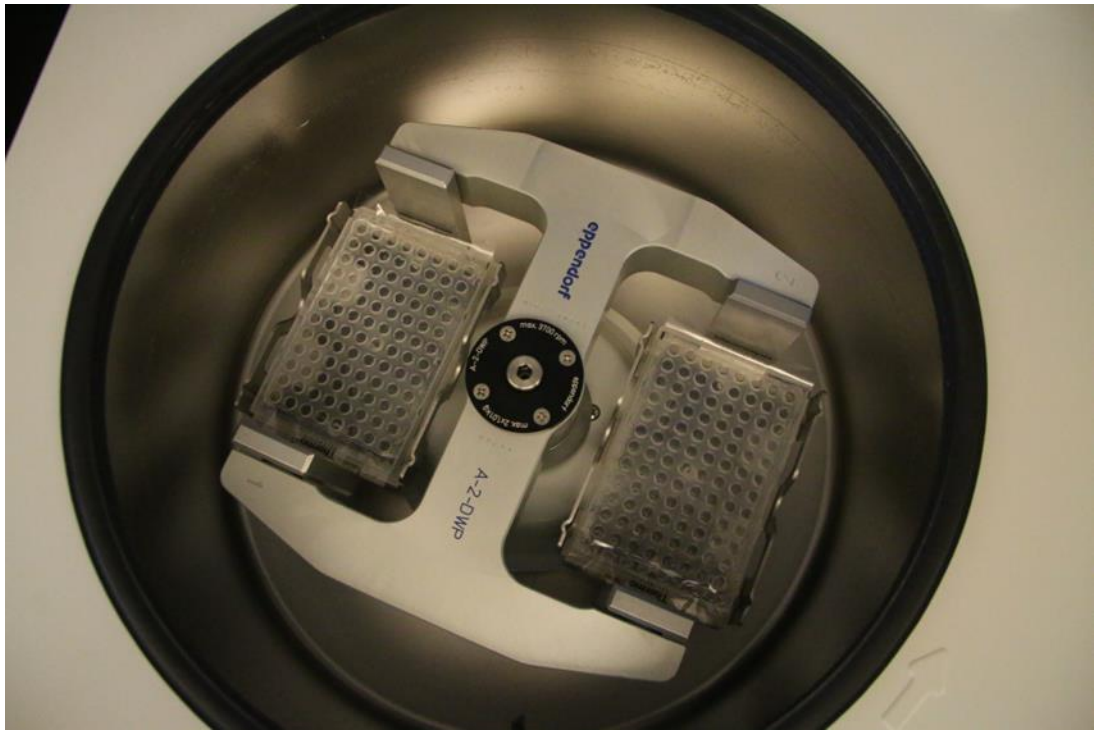


- Aliquot 3 µl genomic DNA into PCR plate
- Aliquot mastermix into PCR plate
- Seal PCR plate for thermocyling

Complete thermocycling for PCR 1

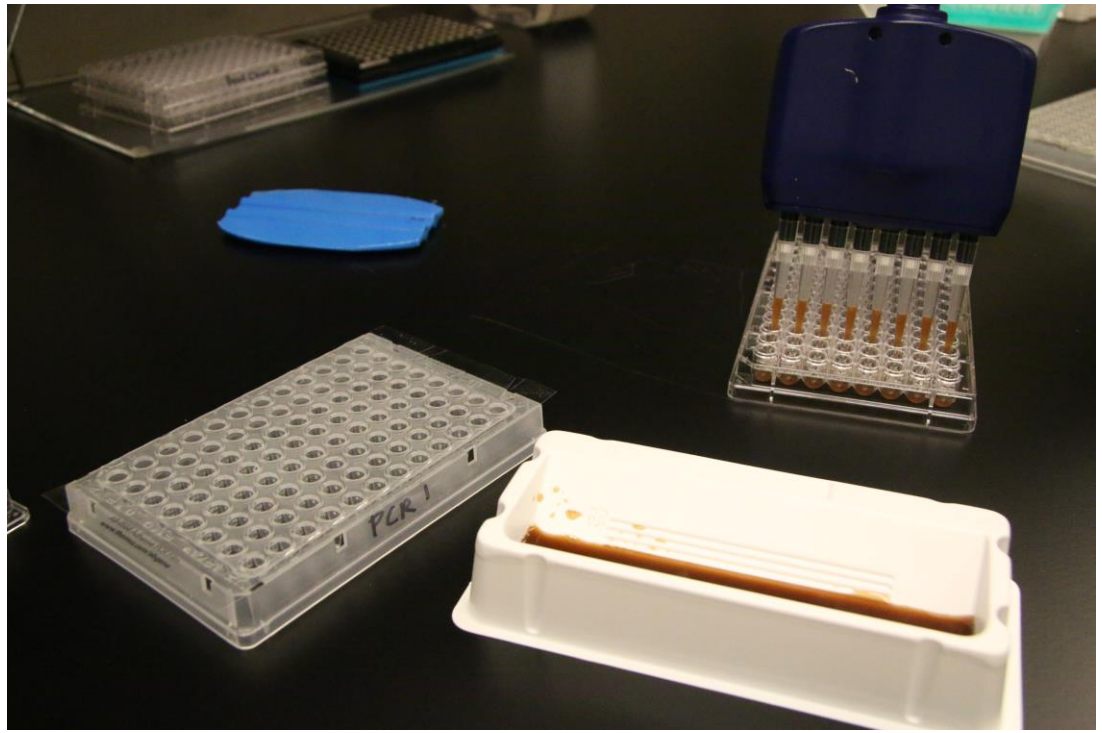
Step number	Number of cycles	Temperature	Time
1	1	98°C	30 sec
2	30	98°C	10 sec
3		60°C	30 sec
4		72°C	1:30 min
5	1	72°C	6:00 min
6	1	4°C	HOLD

Centrifuge PCR plate after thermocyling



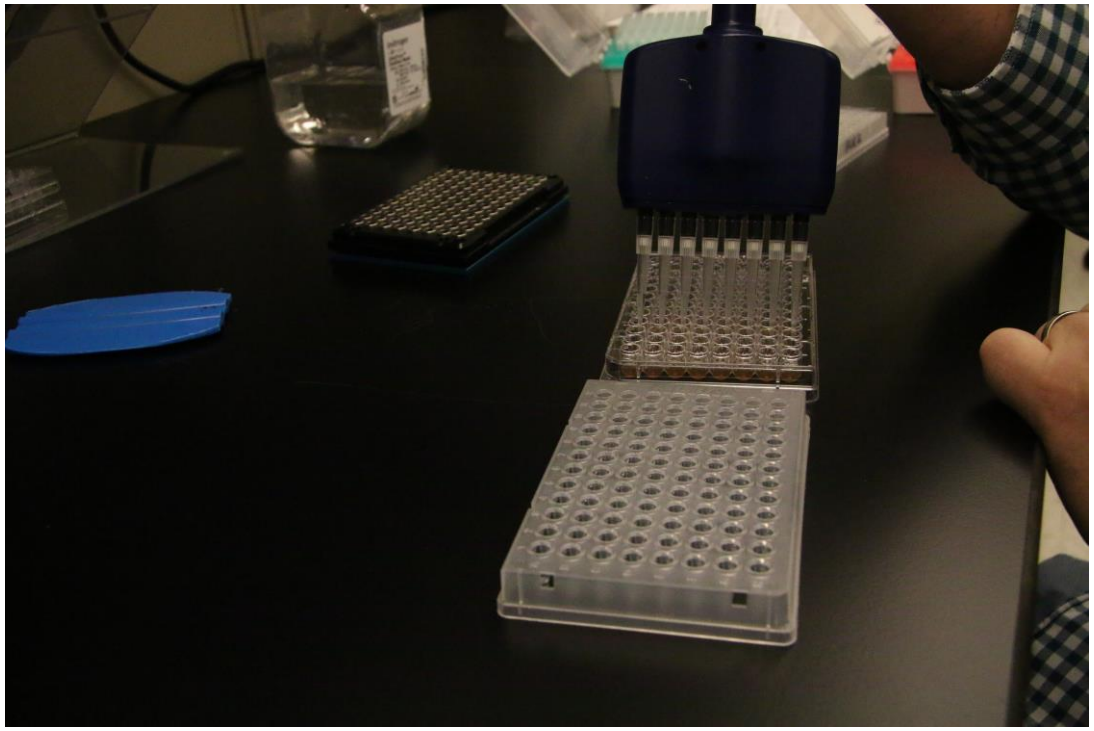
- Perform a quick spin of PCR plates after cycling to pull all liquid to the bottom of the wells

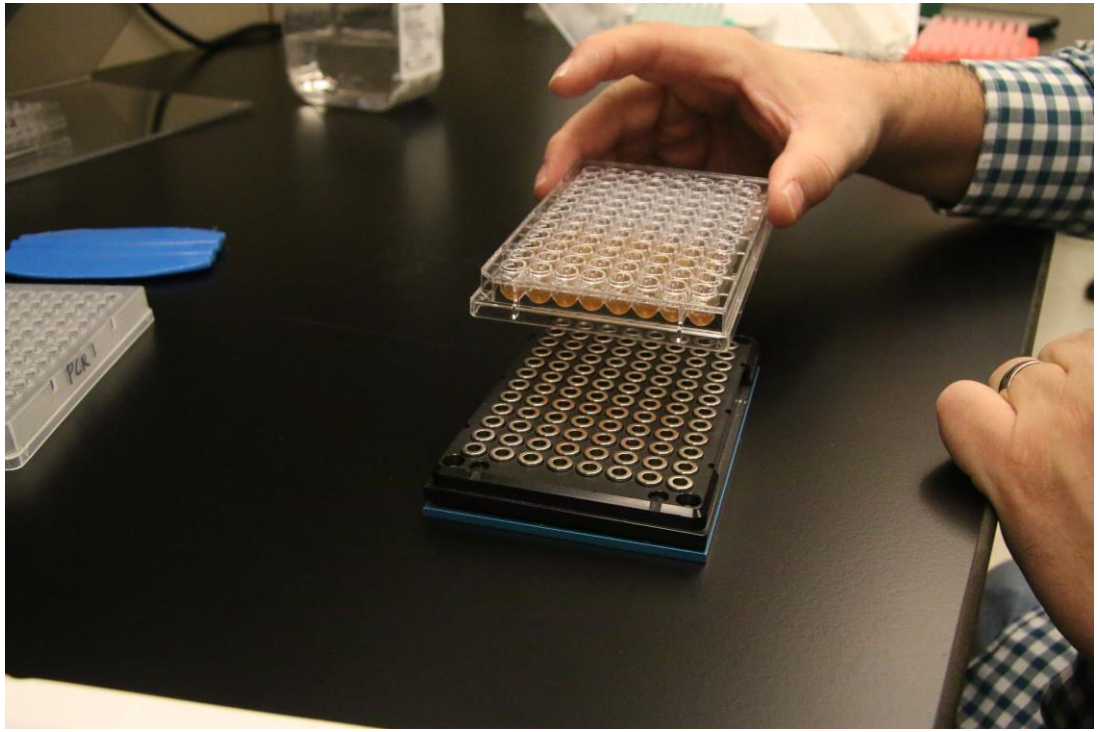
Prepare PCR 1 for bead cleanup



- Ensure BioChain® SeqPure beads are equilibrated to room temperature and are thoroughly mixed
- For KAPA, add 50 ul of beads per sample
- For Phusion, add 40 µl of beads and 20 µl water per sample
- Add beads to a new 96-well round-bottom plate

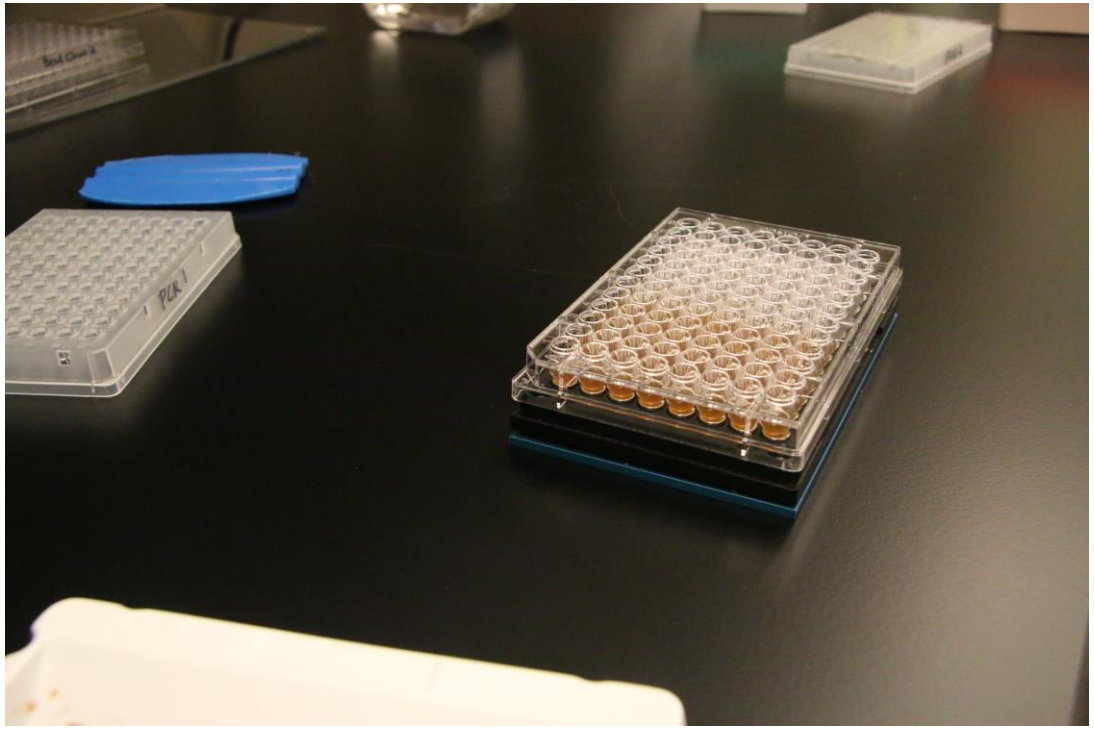
Pulling out the magnetic beads in cleanup



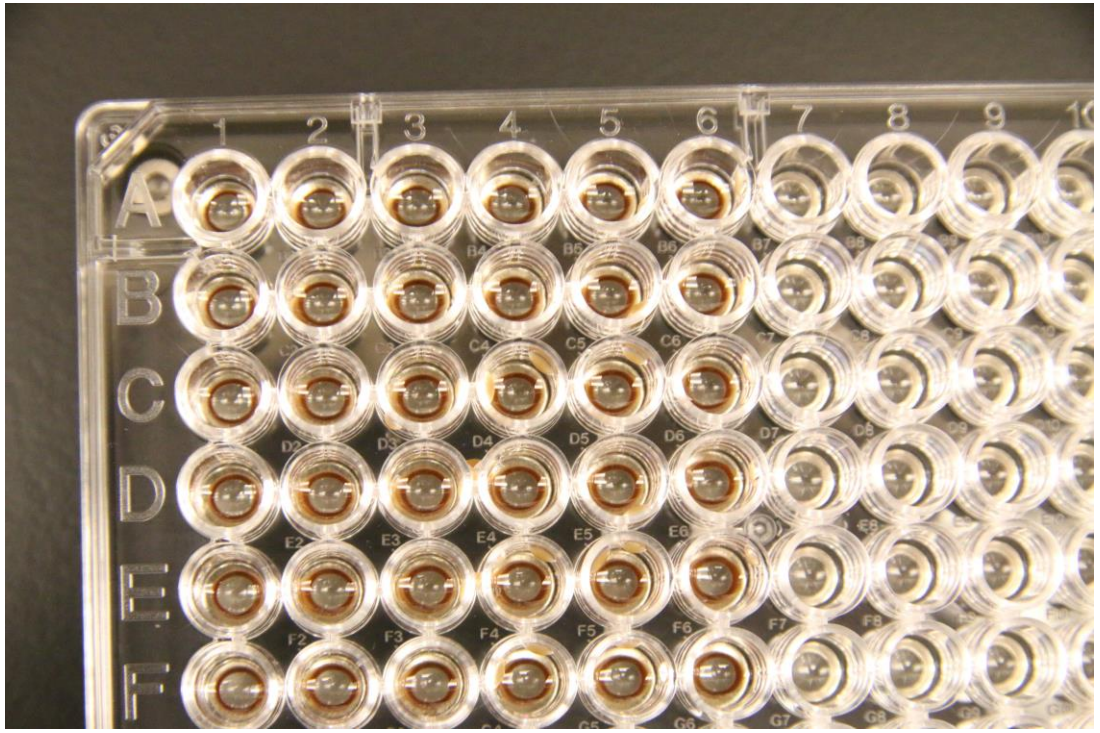


- Add entire PCR 1 product to plate with magnetic beads and mix thoroughly
- Incubate at room temperature for 5 minutes
- Place plate on magnetic stand until liquid appears clear

Pulling out the magnetic beads in cleanup continued

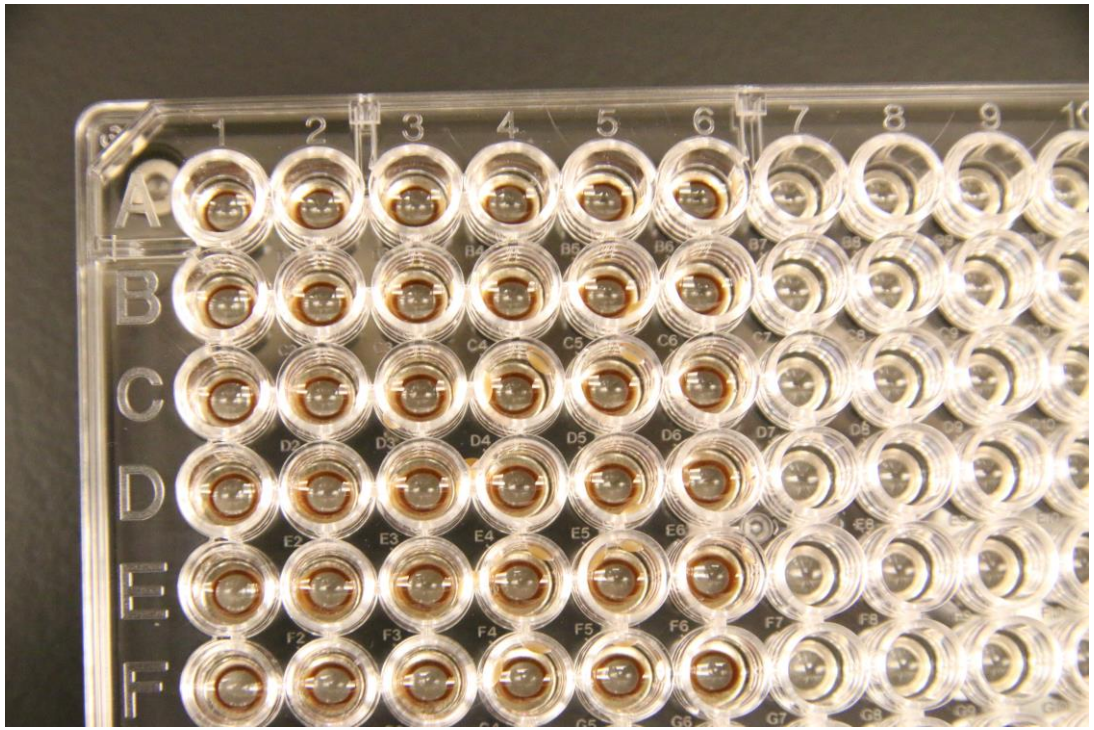


Separation of beads from supernatant

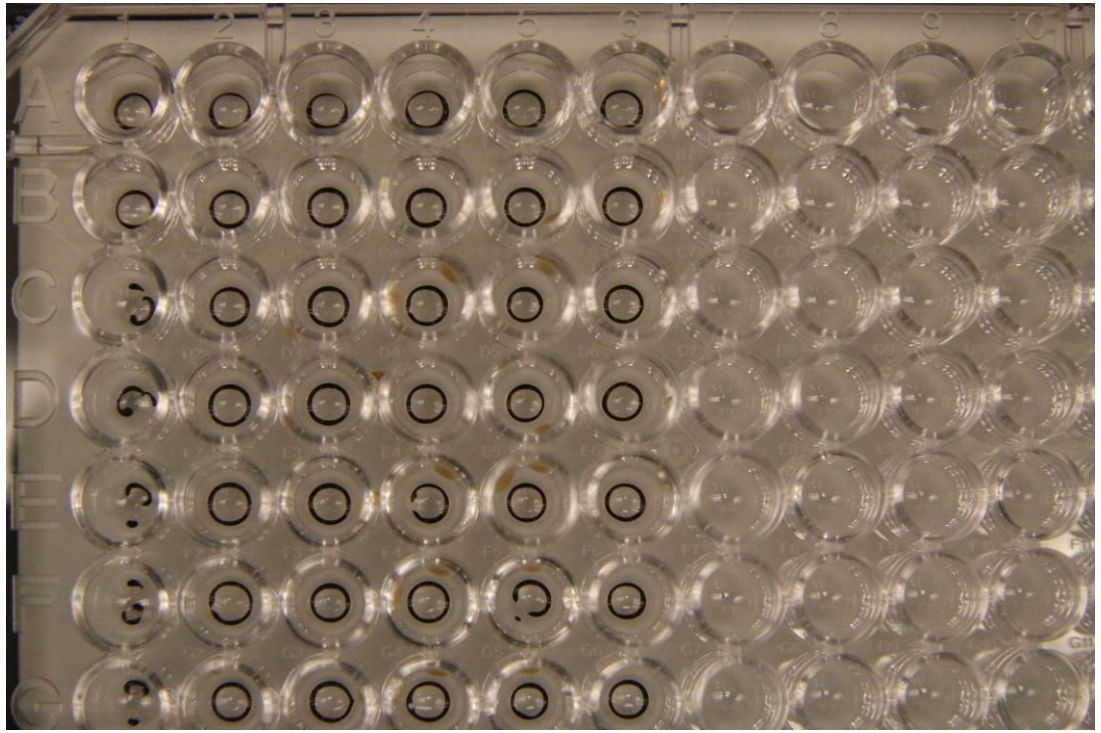


The magnet will create circles of beads at the bottom of the wells, indicating separation of the beads

Supernatant removal following bead separation

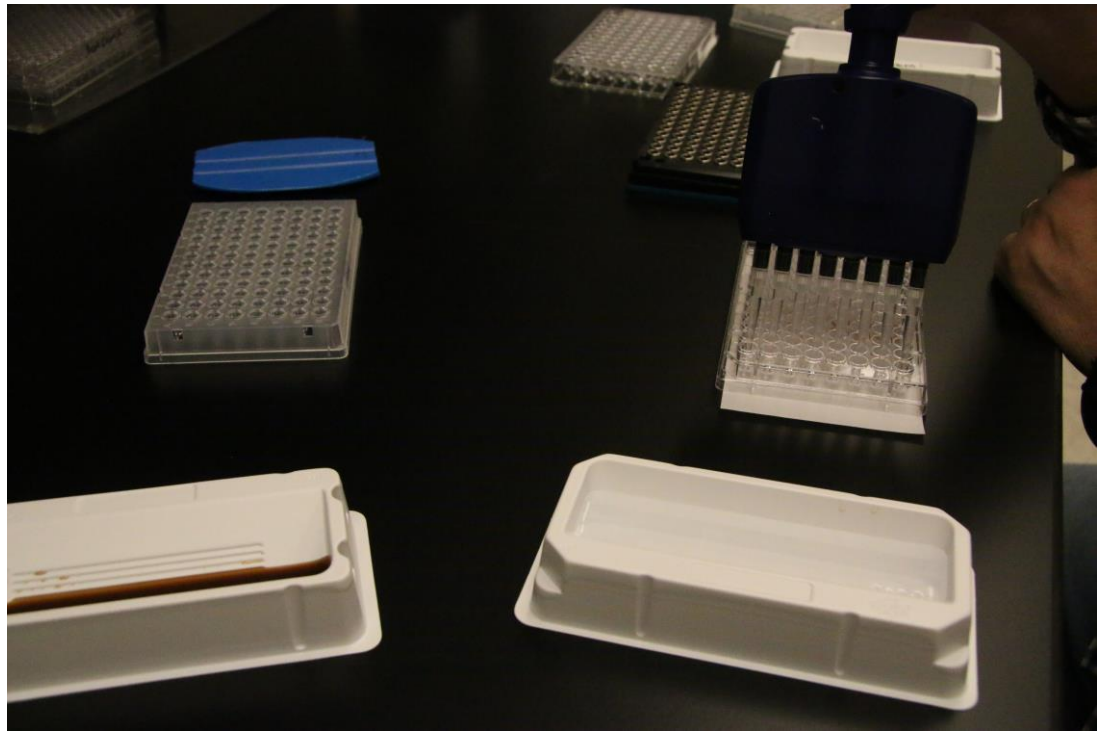


Separated beads from supernatant, ready for supernatant removal



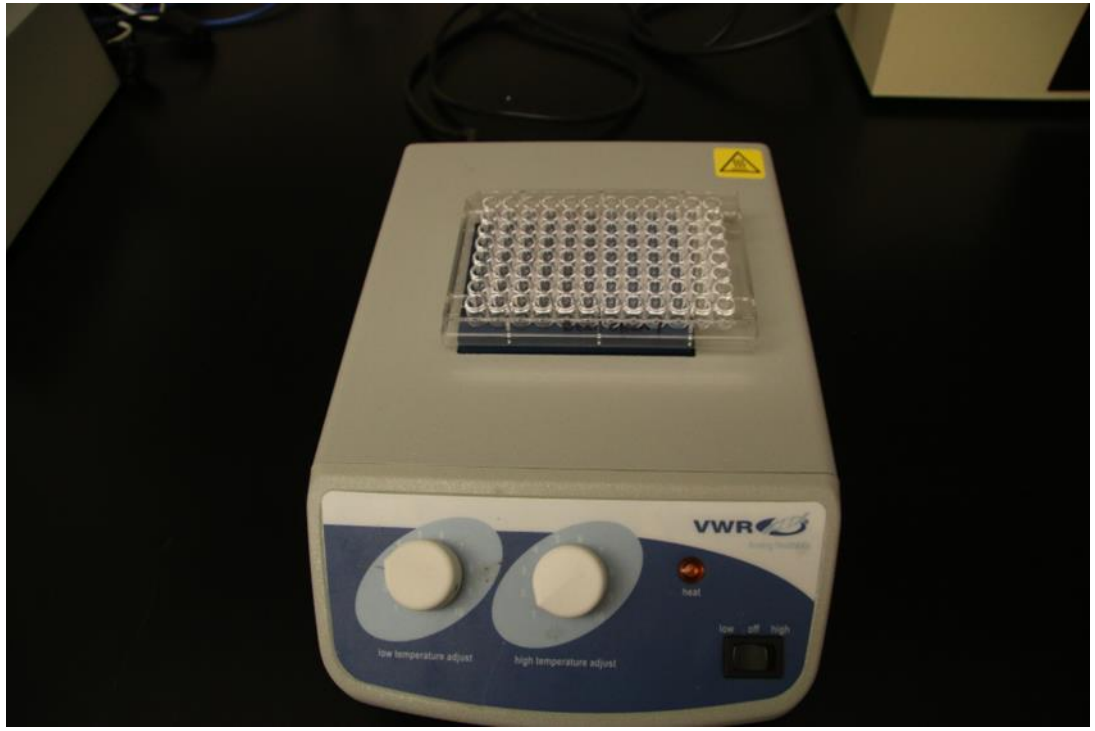
- Use pipette to remove supernatant from wells, and discard the supernatant
- You will see a black ring at the bottom of the well, which are the separated beads, ready for wash step

Ethanol wash of beads from PCR 1



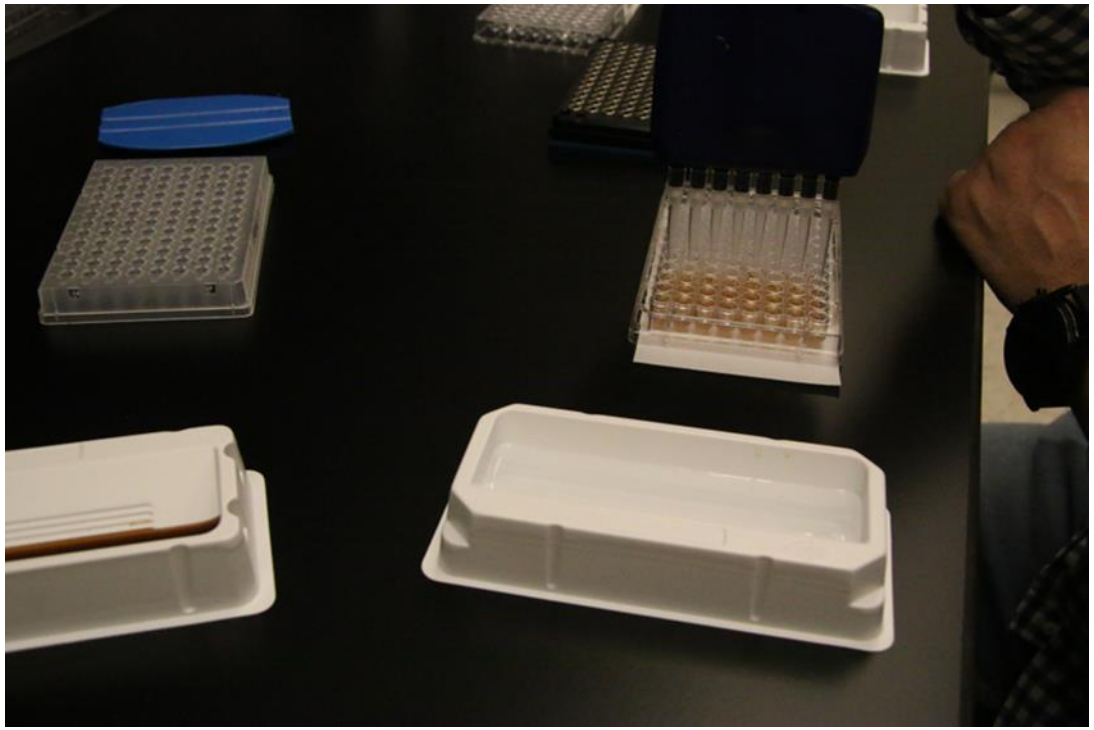
- Add 200 µl of 80% ethanol to wells
- Let stand for 30 seconds, then discard the supernatant, taking care not to disturb the beads
- Repeat this ethanol wash for a second time

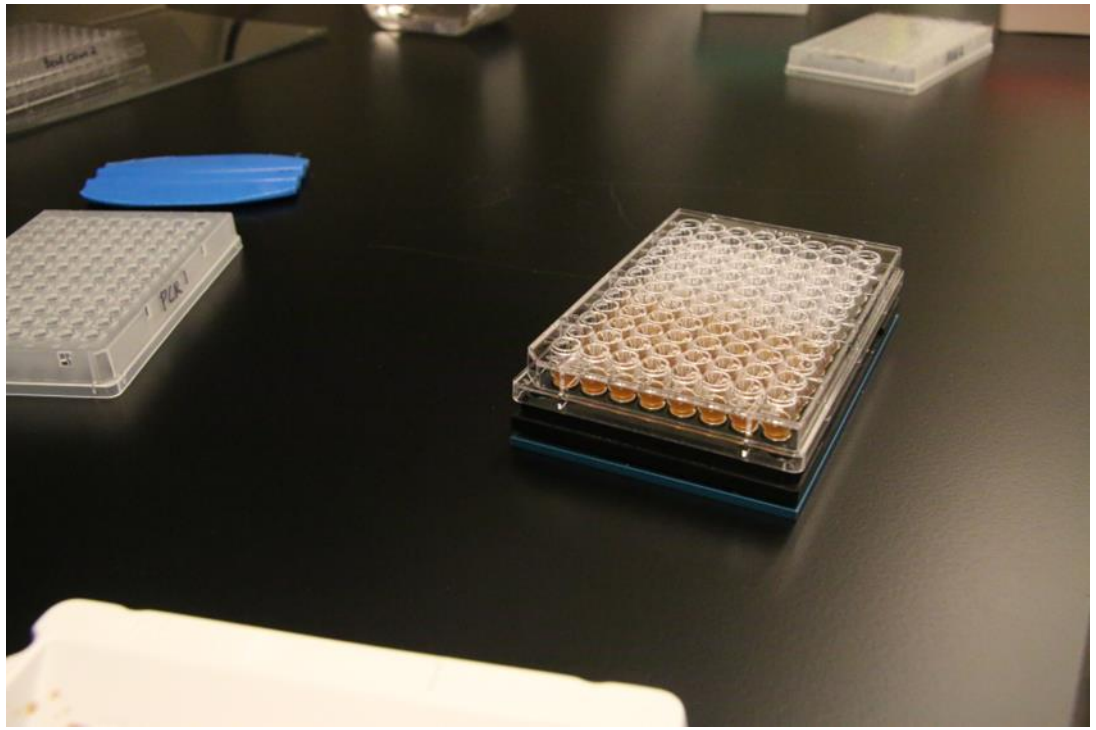
Dry down of beads following bead wash



- Dry beads on a heat plate at 37^{o}C until dry (beads may appear cracked)

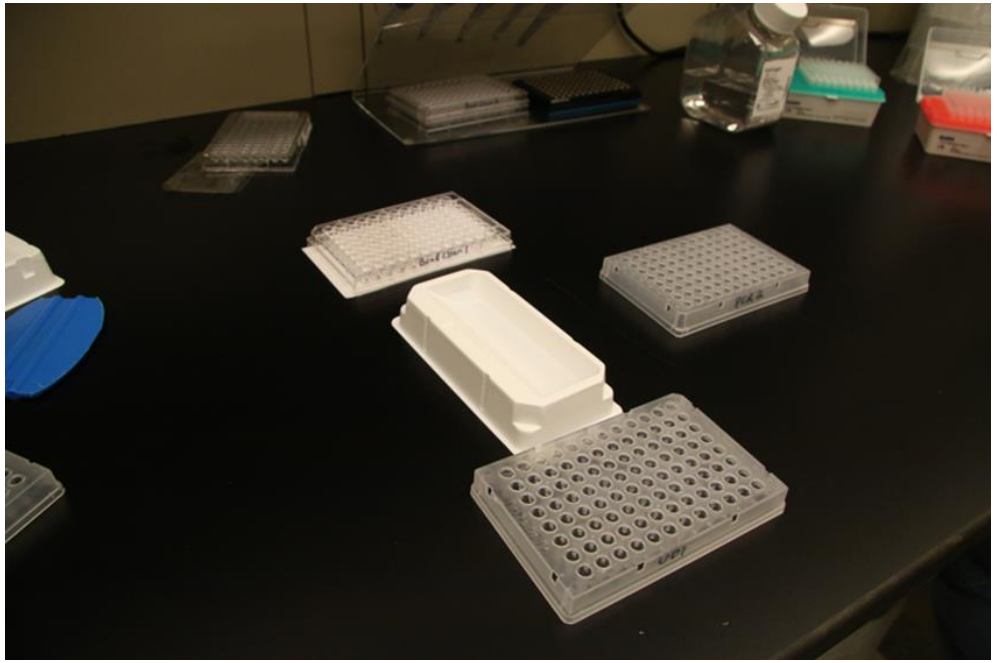
Elution of beads in preparation for PCR 2





- Resuspend beads with 25 µl elution buffer, gently pipette up and down to mix
- Incubate plate for 5 minutes at room temperature
- Place plate on magnetic plate at room temperature until liquid is clear
- Transfer 20 µl to a new PCR plate

Prepare PCR2 mastermix



Reagent	Concentration	Volume
KAPA Mastermix	2x	25ul
UltraPure Water		1 ul

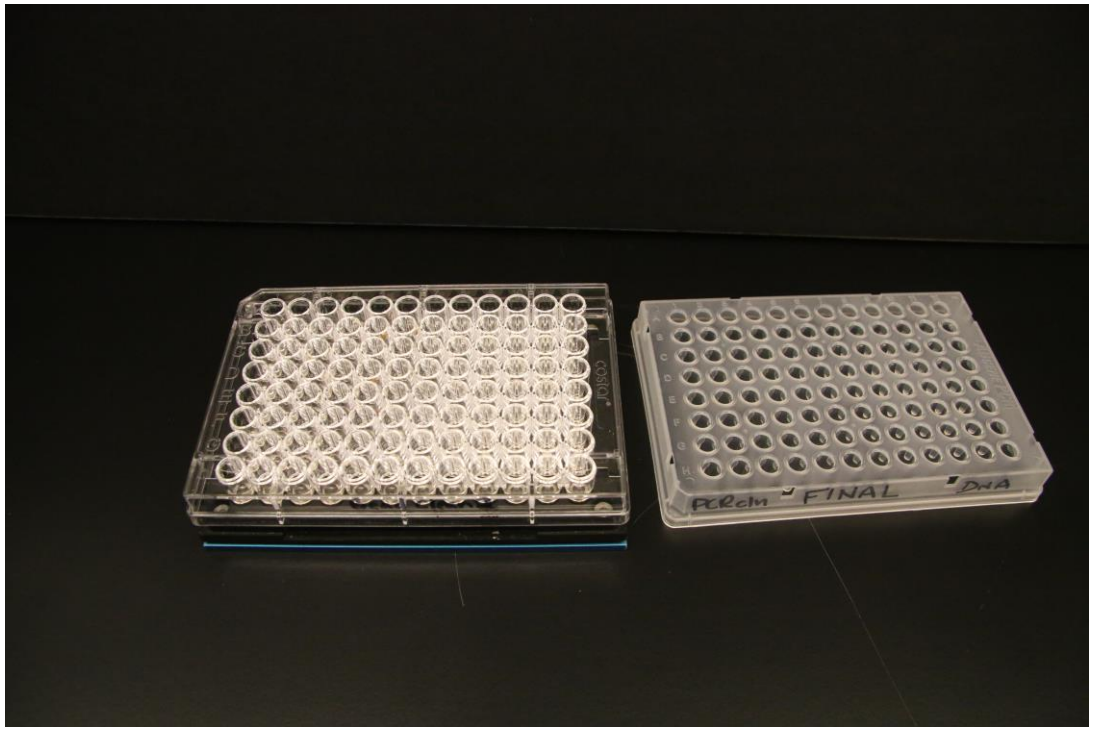
Volumes are per sample

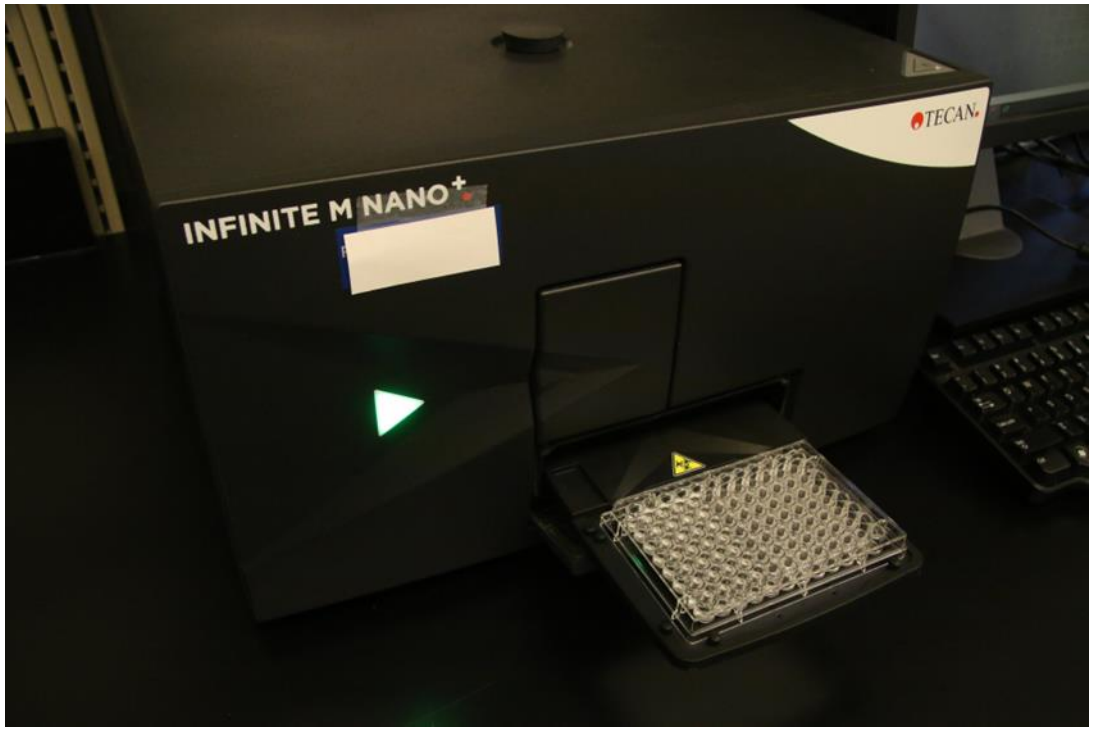
- Add 3 µl of Unique Dual Index adapter primers (1 µM) or equivalent to each sample
- Add 26 µl of each PCR mastermix to each sample and mix thoroughly with pipetting
- Seal with adhesive seal

Complete thermocycling for PCR 2

Step number	Number of cycles	Temperature	Time
1	1	72°C	3 min
2	6	98°C	10 sec
3		63°C	30 sec
4		72°C	3 min
5	1	4°C	HOLD

Bead cleanup and DNA quantification following PCR 2

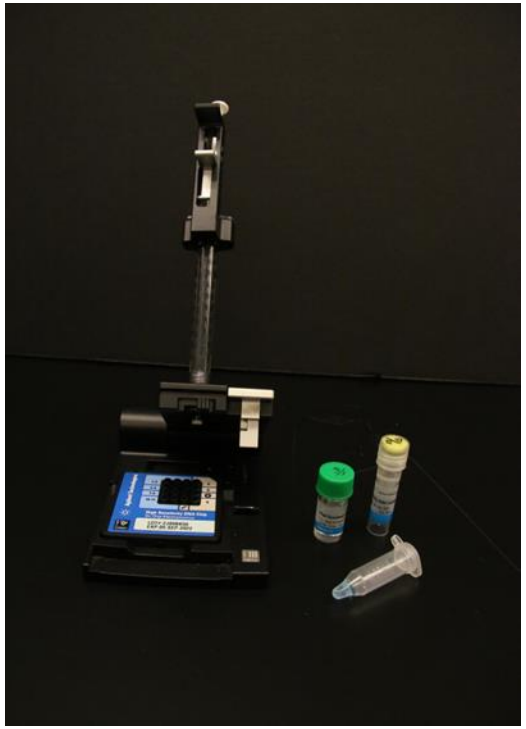




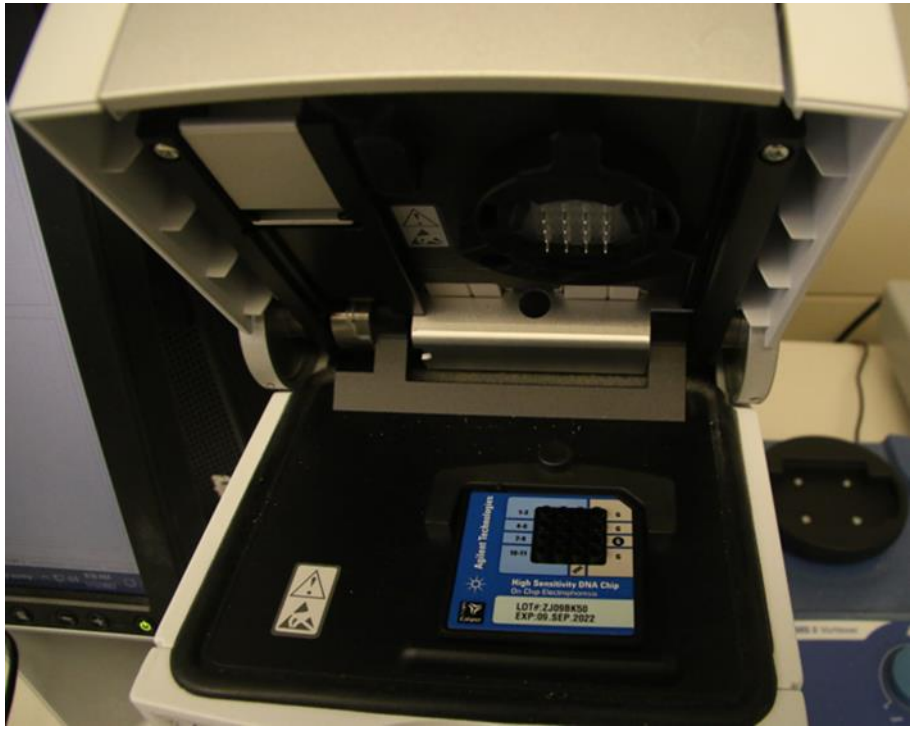
- Repeat the same PCR cleanup steps following PCR 2 as used for PCR 1 bead cleanup
- Elute cleaned amplicons with 25 µl elution buffer amplicons into 96-well plate

- Perform Pico Green DNA quantification of PCR amplification or equivalent DNA concentration method

Analysis of library amplicon size



Agilent High Sensitivity DNA chip setup



Agilent chip in Agilent Bioanalyzer

- Perform analysis of library to confirm expected size of amplicon
- Other methods such as agarose gel-based visualization can be used
- Samples can be analyzed for sequence by Illumina-based sequencing

Further reading on gene editing detection

Shillito, R.D., Whitt, S., Ross, M. *et al.* Detection of genome edits in plants—from editing to seed. *In Vitro Cell.Dev.Biol.-Plant* **57**, 595–608 (2021). https://doi.org/10.1007/s11627-021-10214-z

Lomov, N.A., Viushkov, V.S., Petrenko, A.P. *et al.* Methods of Evaluating the Efficiency of CRISPR/Cas Genome Editing. *Mol Biol* **53**, 862–875 (2019). https://doi.org/10.1134/S0026893319060116

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Thank you!

Any questions?