SCST Genetics Super Workshop

PCR Set Up and Troubleshooting

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PCR Technique

- Developed in 1984 and 1985 by Mullis, Saiki, Scharf, Faloona, Horn, Erlich, and Arnheim
- *In vitro* method that greatly amplifies DNA sequences that otherwise could not be detected
- Utilized to amplify a given DNA sequence that constitutes less than one part per million of initial sample
- Procedure alleviated the necessity of *in vivo* replication of a target DNA sequence or of replication of one-of-a-kind tiny DNA samples

Basic PCR Components Needed

- Reagents:
  - Template DNA
  - Mastermix
    - Ready to use solution for efficient amplification
    - TAQ DNA Polymerase: A 94 kilodalton DNA polymerase, which was originally isolated from the thermophilic bacteria *Thermus aquaticus.* Commonly utilized to catalyze PCR reactions due to heat resistance
  - Primers
    - Could be referred to as “oligo”
    - Short single stranded DNA sequence that complements the target sequence
  - Probes
    - Specific labelled target DNA sequence
- Consumables
  - Plates or Tubes
  - Seal

Basic PCR Components Needed (cont'd)

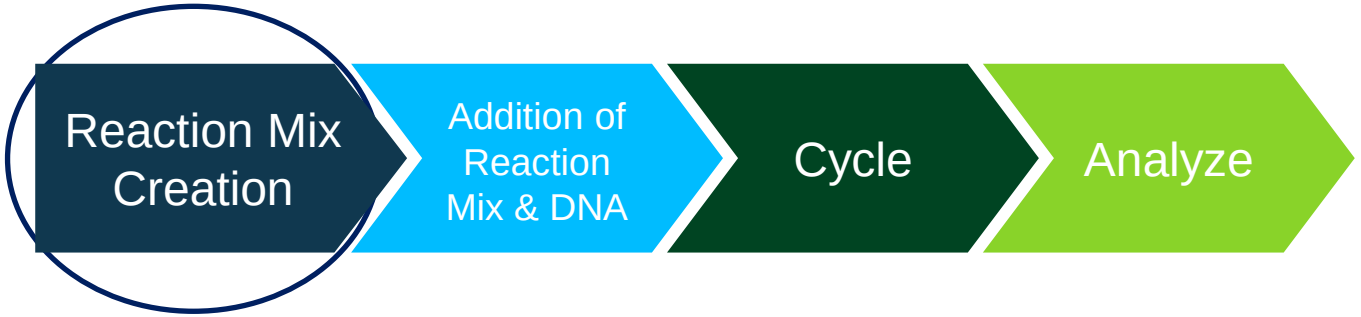
- Liquid Handling Device
  - Manual:
    - Pipette
      - Single
      - Multi-channel
  - Semi-Automated:
    - Sorenson Benchtop 96 Well Pipettor
  - Automated:
    - Beckman FX/i5
    - Bionex Hive
    - LGC Nexar

Basic PCR Components Needed (cont'd)

Cycler

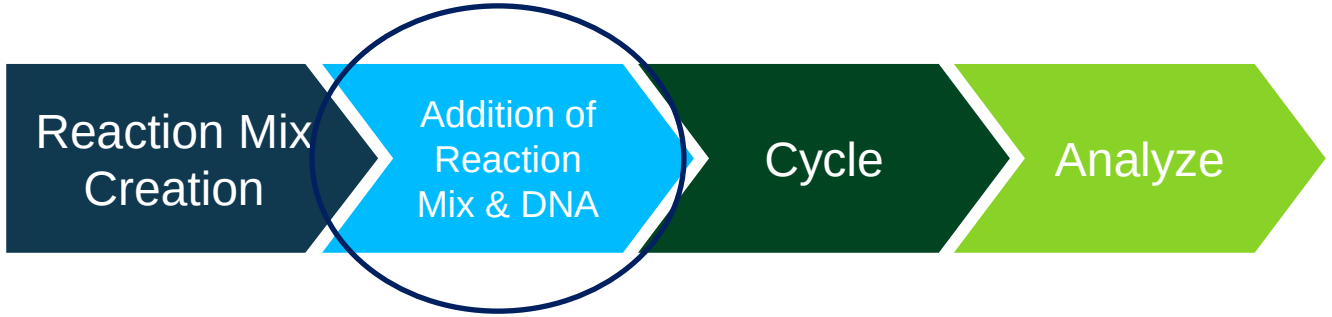
- Hydrocycler
  - Different sizes available
- Block thermocycler
  - Realtime

Reaction Mix Creation



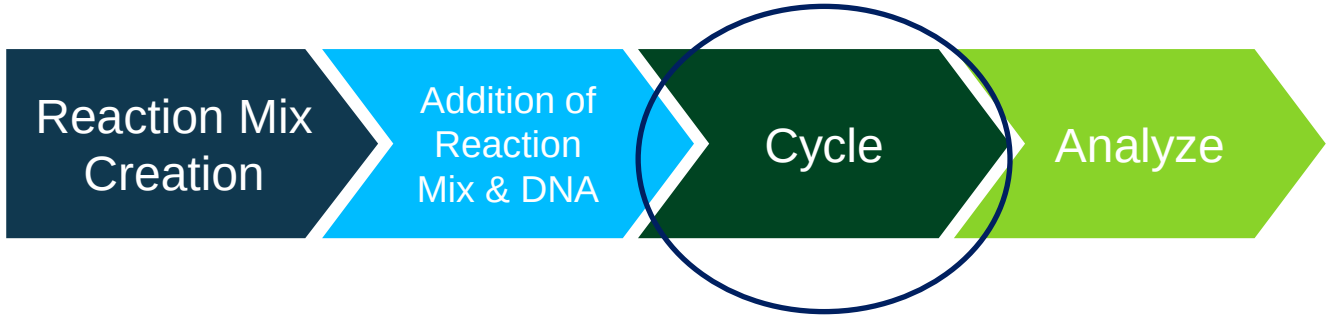
- Determine number of reactions needed
  - Example: 384 well plate with overage: 423 reactions
- Add all components
  - Master Mix, Primers, Probe(s)
    - Different background dyes: ROX, Mustang Purple, ATTO

Addition of Reaction Mix and DNA



- Add reaction mix to plate
- Add extracted DNA to plate
  - Utilize liquid handlers or perform manually for each task

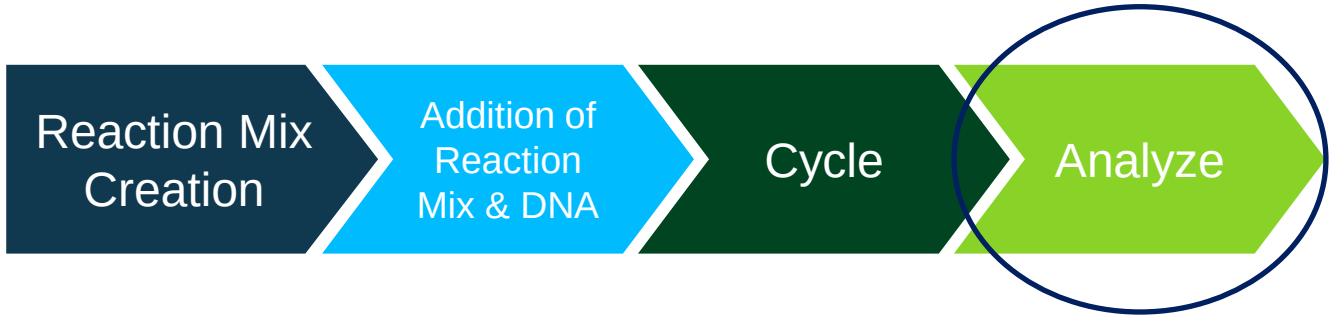
Cycle



Stage	Step	Temperature	Time
Holding	DNA Polymerase Activation	95ºC	20 sec
Cycling (40 cycles)	Denature	95ºC	15 sec
	Anneal/Extend	60ºC	60 sec

Adapted from ThermoFisher TaqMan GTXpress Master Mix Protocol

Analyze



Analysis will be determined by the type of technology used.

- All reactions discussed above imply a fluorescent TaqMan reaction via plate reader

Reactions could also be analyzed using gel technology.

Troubleshooting

Troubleshooting

Adding Controls

- Gives you something to compare your samples to
- Adding to extraction plate will give better picture of what failed in the process
- Adding to assay plate will tell you if your assay failed
- Assay internal controls can also be used to ensure reaction success
- Give more data confidence

	1	2	3	4	5	6	7	8	9	10	11	12
A	Positive Control	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
B	Positive Control	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
C	Positive Control	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
D	Positive Control	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
E	Negative Control	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
F	Empty	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
G	Negative Control	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample
H	Empty	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample	Sample

Troubleshooting

QC Assays

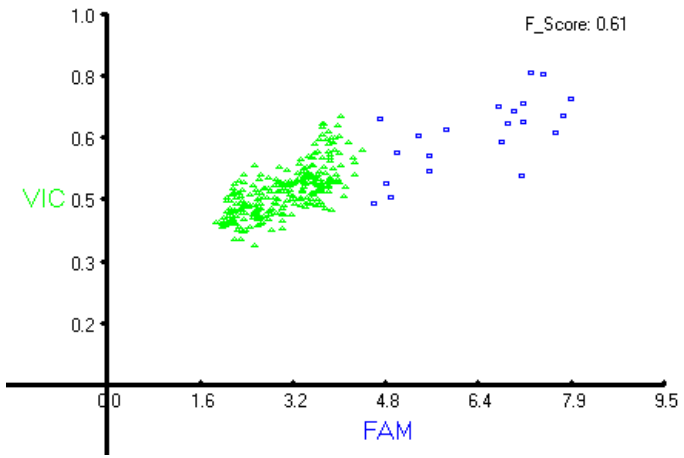
- Performing a quality test on assay before use will ensure components have been added properly
  - Especially useful when making bulk reaction mixes
- Assurance if your samples have poor performance
  - Eliminating variable

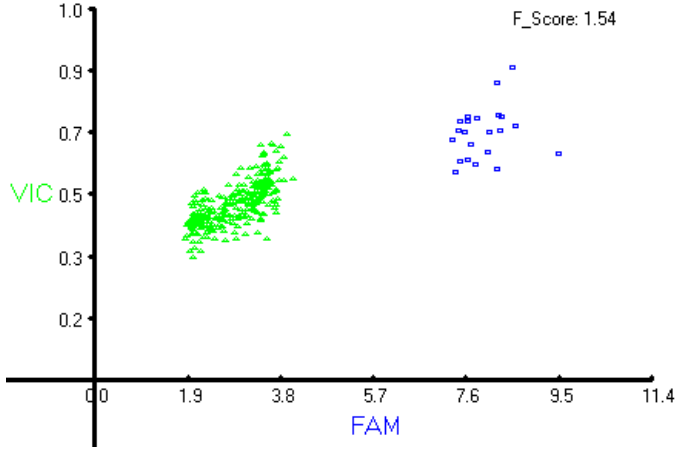
	1	2	3	4	5	6	7	8	9	10	11	12
A	POS	POS	POS	NEG	NEG	NEG	POS	POS	POS	No DNA	No DNA	No DNA
B	POS	POS	POS	NEG	NEG	NEG	POS	POS	POS	No DNA	No DNA	No DNA
C	POS	POS	POS	NEG	NEG	NEG	POS	POS	POS	No DNA	No DNA	No DNA
D	POS	POS	POS	NEG	NEG	NEG	POS	POS	POS	No DNA	No DNA	No DNA
E	POS	POS	POS	NEG	NEG	NEG	POS	POS	POS	No DNA	No DNA	No DNA
F	POS	POS	POS	NEG	NEG	NEG	POS	POS	POS	No DNA	No DNA	No DNA
G	POS	POS	POS	NEG	NEG	NEG	POS	POS	POS	No DNA	No DNA	No DNA
H	POS	POS	POS	NEG	NEG	NEG	POS	POS	POS	No DNA	No DNA	No DNA

Troubleshooting

Adding Additional PCR Cycles

Can be placed back in thermocycler for additional cycles especially for poor separation.





Stage	Step	Temperature	Time
Cycling (10 Cycles)	Denature	95ºC	15 sec
	Anneal/Extend	60ºC	60 sec

Adapted from ThermoFisher TaqMan GTXpress Master Mix Protocol

Troubleshooting

Contamination

- Contaminant: Any unwanted or undesired organism, compound, or molecule present in a controlled environment. Unwanted presence of an entity in an otherwise pure or clean environment.
- Can be seen with an excessive amount of false “positives”
- Where using standards can be useful
- Redilute is a good option to pinpoint where contamination occurred

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	POS		NEG		POS		POS		NEG		NEG		POS		NEG		NEG		NEG		NEG		NEG	
B																								
C	POS		NEG		NEG		POS		NEG		NEG		POS		POS		NEG		NEG		NEG		NEG	
D																								
E	POS		POS		NEG		NC		POS		NEG		POS		NEG		NEG		POS		NEG		NEG	
F																								
G	POS		POS		POS		POS		POS		NEG		NEG		NEG		NEG		POS		NEG		NEG	
H																								
I	POS		POS		POS		POS		NEG		NEG		NEG		POS		POS		NEG		POS		POS	
J																								
K	POS		POS		POS		POS		POS		NEG		POS		POS		NEG		POS		NEG		NEG	
L																								
M	POS		NEG		POS		POS		NEG		NEG		POS		POS		POS		NEG		POS		NEG	
N																								
O	POS		POS		POS		NEG		NEG		NEG		NEG		POS		POS		POS		NEG		NEG	
P																								

Troubleshooting

Transfer/Mix Issues

- Missed wells
  - Master mix
  - DNA
- Components not combined well before transfer

Troubleshooting

Other Considerations

- Assay redesign
  - Establish new primer/probe sequences within the same target region
- Marker concentrations
  - Adjust amounts of primers, probes, master mix in your final total volume

Hands On

Hands On

Determine your amount of reaction mix based on your given calculator

X

Date:	Analyst:

Stock Reagents	Lot number(s)	Volume (ul) needed for each well	Volume (ul) to add to Bulk Reaction Mix
dH2O		1	
Master mix		1.875	
Forward Primer (200uM)		0.05	
Reverse Primer (200uM)		0.05	
Probe (100uM)		0.025	
Final Volume		**3**	

Hands On

// Create your mix based on your calculator

// Pipette into 384-well assay plate