DNA extraction & PCR Protocols
using blastocyst DNA from CRISPR injections

To determine the cutting efficiency of the selected guides, we typically run a nested PCR and send the products for sanger sequencing.

DNA Extraction from blastocysts – our GEM facility will provide the blastocysts

1. Blastocysts are stored in 5ul of QuickExtract buffer (Epicenter QE09050)
2. (** This step has not been critical for yielding high quality sequences**) Add 0.3ul of 1ug/ul yeast tRNA (Thermofisher Scientific #65001) to the 5ul of blastocyst sample.
3. (**Typical starting point for investigators **) Extract DNA by heating tubes in PCR machine
    a. 65˚C for 6min
    b. 98˚C for 2min
    c. hold at 4˚C in a PCR machine.
4. Long term storage at -20oC

Genotyping Notes and Suggestions

- Typically, two rounds of PCR amplification are necessary to amplify the product from the limited blastocyst DNA, although some better primers are able to successfully amplify after one round. During testing it is useful to run some of the first round of PCR amplification on a gel to assess band strength and quality.
- A nested PCR structure is recommended in most cases.
    a. Typically, we design our outer primers to amplify a region of ~700-1000bp and the interior primers to amplify a region of ~350bp
    b. Multiple primer sets may be necessary to produce an optimal sequencing product during testing stages.
    c. Use NEB's Tm calculator to determine Tm for your primers.
- Gel purify the 2nd PCR products (we use ZymoClean) and send for sequencing.
    a. Interior primers should be used when submitting samples for sanger sequencing.
    b. We generally submit both Forward and Reverse primers to improve the likelihood of catching cutting events.
    c. Sequencing products should be analyzed for cutting in the expected region of sgRNA activity. GEM can help with the analysis if necessary.
- For the PCRs we have tried so far, whenever we see unspecific bands, we dilute the first PCR product 1:10 and use 1ul from this dilution as a template for the 2nd PCR.
- GEM uses Q5 High Fidelity DNA Polymerase (NEB M0491S) with the below PCR cycle protocol
    a. We have had success with other taqs in the past, so it is possible to produce quality sequences with other polymerases, this requires following the suggested protocols from your taq of choice.

Component	25 µl Reaction	Final Concentration
Q5 High-Fidelity Buffer 5x	5.0 µl	1X
10 µM F and R Primers	1.25 µl	0.5 µM
10mM dNTPs	0.5 µl	0.5 µM
Q5 enzyme	0.25 µl	
Nuclease-Free Water	17.0 µl	
Template DNA	1.0 µl	< 1,000 ng

STEP	TEMP	TIME
Initial Denaturation	98°C	30 seconds
25–35 Cycles	98°C	10 seconds
	Primer's TM	15 seconds
	72°C	15 seconds (20–30 seconds/kb)
Final Extension	72°C	2 minutes
Hold	4–10°C	

Suggested Blastocyst PCR for initial genotyping strategy

- Since the blastocysts are composed of a limited number of cells, developing a working PCR can be challenging
- Several rounds of temperature, primer and other condition optimization may be necessary before performing genotyping on experimental blastocysts
- The following is a recommended combination of samples to test the quality of the genotyping PCR.
- This PCR should be run and optimized for sequencing before moving onto experimental blastocysts
- GEM can provide WT blastocysts for genotyping optimization
- Samples should be submitted for Sanger sequencing and analyzed for sequence quality.

Samples:

1. Tail DNA ~250ng/uL
2. Tail DNA ~25 ng/uL
3. Tail DNA ~2.5ng/uL
4. WT Blastocyst sample
5. WT Blastocyst sample
6. Negative Control (water)