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Mouse Blastocyst DNA Extraction and PCR Amplification

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Transgenic Animal Mod...



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Protocol status: Working

We have used this protocol since 2014 and it works great.

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Abstract

The purpose of this protocol is to genotype mouse blastocysts. Mouse blastocysts can be obtained by culturing fertilized eggs in vitro or by in vivo collection from female mice. Single mouse blastocysts contain about 64 cells, yielding a few picograms of genomic DNA. The DNA extraction protocol below is based on Sakurai et al. (2014). The result is a crude DNA extract that is then placed in a PCR reaction to detect genes or genetic changes of interest. Due to low DNA yields from single blastocysts, we use 29 nucleotide long PCR primers to increase specificity of PCR products so that only a single DNA band is amplified from genomic DNA as described (Stratman et al. 2003). We prefer to use a software driven approach to primer selection rather than guessing at primer sequences. For this purpose, we use the web-based Primer-BLAST tool at NCBI (Ye et al. 2012). Often, we find that GC rich DNA templates amplify with low efficiency. The addition of CES (Combinatorial PCR Enhancer Solution) to the PCR reaction mix can improve PCR efficiency of some difficult to amplify templates (Ralser et al. 2006). We use this method to determine if fertilized eggs treated with CRISPR Cas9 or other similar reagents carried designed genetic changes. In this way we can validate CRISPR reagents prior in our CRISPR pipeline before we use large numbers of mice to generate a novel mouse model for translational preclinical research.

Safety warnings

- ! All activities involving animals require approval by an IACUC or other relevant regulatory body prior to executing this protocol.

Ethics statement

All animal activities described were reviewed and approved by the University of Michigan IACUC.

Mouse Blastocyst DNA Extraction and PCR Amplification

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Table 1. List of Materials for Blastocyst DNA Extraction and PCR.

	A	B	C
	Individual mouse blastocysts in 10µl H2O in PCR tubes.		Store at -20 or -80°C.
	Pipetman.		
	RNAse Free Pipetman Tips.		
	PCR tubes.		
	Nuclease Free Water.	SigmaAldrich Cat. no. W4502.	
	Blastocyst Lysis Buffer.	2X	
	Gene specific PCR primers.	29 nucleotides in length.	
	Hot Start Taq DNA polymerase of choice.		
	Taq DNA polymerase buffer.	10X	
	Thermal Cycler for PCR.		
	1M Tris-HCl, pH 8.3.	Teknova Cat. no. T5083	
	1 M KCl.	SigmaAldrich Cat. no. 60142.	



A	B	C
2% Gelatin solution.	SigmaAldrich Cat. No. G1393.	
Tween 20.	SigmaAldrich Cat. no. P9416.	
10 mg/ml yeast tRNA in nuclease free water.	SigmaAldrich Cat. no. R5636.	Store at -20°C.
20 mg/ml Proteinase K.	New England BioLabs Cat. no. P8107S.	Store at -20°C.
5M Betaine solution.	SigmaAldrich Cat. no. B0300.	
1M DTT (DL-Dithiothreitol) solution.	SigmaAldrich Cat. no. 43816.	
DMSO.	SigmaAldrich Cat. no. D8418.	
35% BSA solution.	SigmaAldrich Cat. no. A7979.	

2 Prepare Reagent: Blastocyst Lysis Buffer Base.

For 10 ml of solution combine the reagents as shown in the table:

Store at room temperature.

A	B
1 M Tris-HCl (pH8.3).	2.0 ml.
1 M KCl.	2.0 ml.
2% gelatin.	40 µl.
Tween 20.	90 µl.



A	B
Water.	5.87 ml.

3 Prepare Reagent: 2X Blastocyst Lysis Buffer.

For 1 ml of solution combine the reagents as shown in the table:

Store at -20°C.

A	B
Blastocyst Lysis Buffer Base.	1.0 ml.
10 mg/ul Yeast tRNA.	6.0 µl.
20 mg/ml Proteinase K.	6.25 µl.
Water.	

4 Prepare Reagent: 5X CES (Combinatorial PCR Enhancer Solution).

For 1 ml of solution combine the reagents as shown in the table:

Store at -20°C.

A	B
5M Betaine.	540 µl.
1M DTT.	6.7 µl.
DMSO.	67.0 µl.
35% BSA.	157.1 µl.
Water.	229.2 µl.

5 Procedure: Blastocyst Lysis.

The blastocyst lysis procedure produces a crude DNA preparation from individual blastocysts.

Add 10µl of 2X Blastocyst Lysis Buffer to each blastocyst frozen in a PCR tube.

Place PCR tubes in the thermal cycler.



Run the following program to prepare the crude DNA solution: 56°C for 10min, 95°C for 10min, 4°C indefinitely.

Store crude lysate at -20°C.

6 Procedure: Blastocyst PCR.

The blastocyst PCR procedure amplifies the DNA segment determined by the primers. Use 4 ul of crude blastocyst DNA in a 20 ul PCR reaction to detect the gene of interest. Add suggested concentrations of primers, Taq polymerase, PCR reaction buffer, and water to 20 ul.

Run the PCR and examine the gel products on a 1% agarose gel.

For example: 98°C for 2 minutes for initial denaturing, Tm -2°C for annealing for 30 seconds, 72°C for extension for 30 seconds, 95°C for subsequent denaturing steps, 35-40 cycles then hold at 4°C indefinitely.

nota bene; We have had good results with KOD polymerase when other Taq polymerases didn't amplify.

note bene; For inefficient PCR reactions, Ralser et al. (2006) further recommend that the PCR reaction buffer contain the following final concentrations of components: 65 mM Tris-HCl, 16.6 mM (NH₄)₂SO₄, 3.1 mM MgCl₂, and 0.01% (v/v) Tween 20 at a pH of 8.8.

7 Procedure: Primer Design for Specific and Sensitive PCR Assays.

If the first primer pair you pick does produce an amplification product, order a new primer pair.

Use Primer-Blast to pick primers <http://www.ncbi.nlm.nih.gov/tools/primer-blast>

Adjust Primer Parameter default settings.

- Minimum primer melting temperature: change to 60°C.

- Optimal primer melting temperature: change to 63°C.

- Maximum primer melting temperature: change to 66°C.

- Minimum primer melting temperature difference: change to 1°C.

Adjust Primer Pair Specificity Checking Parameters

- Click box to turn on "Enable search for primer pairs specific to the intended PCR template"

- Set Search Mode to "Automatic"

- Set Database to "Genomes for selected eukaryotic organisms (primary assembly only)"

- Set Organism to "Mus musculus (taxid:10090)" for mouse.

Open Advanced Parameters

- Change Primer Minimum size to 14

- Change Primer Optimum size to 29

- Change Primer Maximum size to 31.

nota bene. Stratman et al. reported primers of 27-30 nucleotides made up of 50-60% GC content will produce a 100-500 bp PCR product that uniformly detects genomic DNA with single copy sensitivity.

8 Procedure: PCR Product Analysis.

The suggested parameters are expected to produce a single band of the expected size on an agarose gel. If no bands are detected on a gel, you may wish to obtain a PCR primer for an endogenous DNA sequence. Optimize those primers in a 50 pg DNA, 20 ul PCR reaction to simulate blastocyst DNA conditions. These primers should then amplify blastocyst DNA. If no amplification occurs it may be that the blastocyst DNA is absent from the reaction due to an empty tube, degraded DNA, or the presence of PCR inhibitors. In addition to this type of positive control, it is also advisable to always run a negative control (water template) to ensure that there are no background contaminating DNA in the PCR reactions.

Protocol references

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