Validation of CRISPR knock in (Rab11s)

Introduction

Here is described sequencing strategy of bulk cell populations sorted for fluorescence (fluorophore knock in). Nested PCR was used to pre-amplify genomic knock in events (specific primers flanking homologous sequences used for integration), followed by gel cutting (the predicted size >1000!) and second PCR (fluorophore and gene specific primers).

OVERVIEW:

Materials

- Primers (their sequences are listed in Excell file)
- 4x Lysis buffer (400 mM Tris pH 8.5, 2% Proteinase K (v/v); 2% Tween20 (v/v);2% Triton-X100 (v/v))
- Reagents and enzymes for PCR (we used PfuX7 compatible with difficult GC rich sequences)
- Agarose gel
- Magnetic beads (Agencourt AMPure XP) + magnetic rack
- Gel extraction columns for DNA

Procedure

Genomic lyzates

1. Seed cells 1 day before lysis. Cells should be 90-100 % confluent (12 well-plate). Wash them 1x with PBS and then add 200 µl of trypsin-EDTA solution
2. Once cells in suspension, transfer 150 µl of cell suspension to 50 µl of 4x Tris lysis buffer (400 mM Tris pH 8.5, 2% Proteinase K (v/v); 2% Tween20 (v/v);2% Triton-X100 (v/v))
3. Incubation 55 C at least 2 hours (it will become clear)

   After first hour invert several times and briefly centrifuge evaporated water back to solution
4. Then heat 95 C 10 min (degradation of proteinase K)
5. Centrifuge 10 min max (pelet of proteinase K at the bottom).. Use for PCR or freeze for further use.

PCR overview:

LIST of primers used:

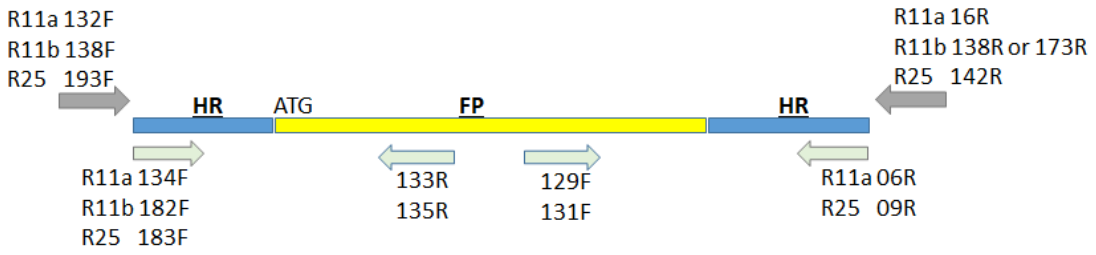

Table2

	A	B
1	Genomic primers	
2	16 R_Rab11A_intron out	AAGCGAAGAACCCGTCAGAG
3	130 R_Rab11A intron in	GAAGGCCTATGGAGAGGTCG
4	132 F_Rab11A intron out	GCTGCCTTCGGCTGTCTAATC
5	134 F_Rab11A intron in	AGTACTTCCCCTTAAAGGCTGG
6	173F_Rab11b_intron out	AGACTGCAACCGAGAAAGGG
7	182F_Rab11b intron in	CCGCTGATAGGCCATTCAAC
8	173R_Rab11b_intron out	CACCTATTGGTCACGCCCAT
9	138 F_Rab11b_intron out	CGTCATCGGGACGTTAAGCA
10	138 R_Rab11b_intron out	GTATAGCCGCGCATCCTAGC
11	183F_Rab25 intron in	TTTGAGAGCTGAGGGTTGAG
12	141 F_Rab25 intron out	GCTCATTAGTTGGGGGACCG
13	142 R_Rab25 intron out	TCTCTCGTTCCCTGTACACCT
14	09 R_Rab25	ACCTCCATCTCTTGTGCTGC
15	193F_Rab25 intron out	CAGTGGGCTGTCTCTGAAGG
16	129 F-mCherry	ACAAGGCCAAGAAACCCGTG
17	133 R-mCherry	CCTTCGCCTTCAATCTCGAACT
18	131 F-NeonGreen	CGCCAACTACCTGAAGAACC
19	135 R_NeonGreen	GGTACTGGTGGAAGCCGTAG

Table1

	A	B	C	D	E	F
1	**Gene**	**1st PCR**	**2nd PCR upstream**	**2nd PCR downstream**	**Sequencing primer upstream**	**Sequencing primer downstream**
2	**mNeonGreen-Rab11a**	132F+16R	134F+135R	131F+130R (or 06R)	135R	131F
3	**mCherry-Rab11a**	132F+16R	132F+133R	129F+16R	134F or 133R	129F
4	**mCherry-R11b**	138F+138R (or 173R)	182F (or 138F) + 133R	129F+138R	133R	129F
5	**mNeonGreen-R25**	193F+142R	183F or (193F) +135R	131F+09R	135R	131F

Note: these or similar combinations (Tm 61 °C or 53)

Genomic PCR

6. Perform PCR as in table bellow, 1 µl of genomic DNA per 20 µl reaction. Run also Ctrl reaction from non-modified cells.

Component	20 µl Reaction	50 µl Reaction	Final Concentration
Nuclease-free water	to 20 µl	to 50 µl	
5X Phusion HF or GC Buffer	4 µl	10 µl	1X
10 mM dNTPs	0.4 µl	1 µl	200 µM
10 µM Forward Primer	1 µl	2.5 µl	0.5 µM
10 µM Reverse Primer	1 µl	2.5 µl	0.5 µM
Template DNA	variable	variable	< 250 ng
DMSO (optional)	(0.6 µl)	(1.5 µl)	3%
Phusion DNA Polymerase	0.2 µl	0.5 µl	1.0 units/50 µl PCR

7. Follow rules... Tm 61 °C or 53. Increase initial denaturation to 2 minutes. 32 cycles

STEP	TEMP	TIME
Initial Denaturation	98°C	30 seconds
25-35 Cycles	98°C 45-72°C 72°C	5-10 seconds 10-30 seconds 15-30 seconds per kb
Final Extension	72°C	5-10 minutes
Hold	4-10°C	

Gel cutting

8. RUN on agarose gel... and cut predicted size (> above 1000!) even if you do not see anything.

   Band corresponding to non-modified allele serves as positive control of PCR and will be always preferentially amplified

9. Purify DNA using gel extraction columns and use eluted product for second nested PCR

   Do not measure on nanodrop as you would probably get zero value (but DNA is there).

Second PCR and purification prior sequencing

10. Run again PCR with different set of primers as in overview, use again 1 µl of template DNA per 20 µl reaction.

11. After the PCR check it on agarose gel, cut the predicted size (and purify it using columns) or if only one correct product you can directly proceed to step 12.

12. Prior sequencing always purify the final product using AMPureXp beads to remove salts and contaminants (ratio 1:1)…. wash 3 times with clean 70 % ethanol/...dry and elute using RNA-free water

13. Measure DNA on nanodrop and send for sequencing