**CRISPR/Cas9-assisted removal of mitochondrial DNA (CARM)**

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**Transformation amplification of plasmid**

100ul DH5a + 10ng plasmid

* In ice incubation for 30min
* 42 oC 1min30s
* In ice 2min 30s

Add 100ul DH5a to 900ul LB medium (without antibody) shaking 45min to recover

Add 1mL recovered bacteria to 250mL LB medium (with Kanamycin 100ug/ml) shaking overnight

Maxi plasmid preparation

**PCR amplification of linear DNA from the plasmid:**

Reaction:

|  |  |
| --- | --- |
| Plasmid | 1ng |
| Phusion buffer (5x) | 10ul |
| T7\_F\_primer | 1ul |
| T7\_R\_primer | 1ul |
| Phusion (Lot 00313012) | 1ul |
| H2O | up to 50ul |

Thermal cycle as follows:

|  |  |  |
| --- | --- | --- |
| 95oC | | 3min |
| 15cycle | 95oC | 30sec |
| 60oC | 30sec |
| 72oC | 1min |
| 72oC | | 5min |
| 4oC | | hold |

* 8 tube PCR reaction per time to ensure the enough product for IVT
* Ampure XP beads Purification (3X)
* In vitro transcription (MEGAshortscriptTM Kit, Thermo Fisher Scientific, Lot AM1354) -- 1ug DNA as input
* sgRNA purification (MEGAclear Kit Purification of Transcription Reactions Lot 00322300)
* sgRNA should be aliquot and stored in -80 oC

**Cas9 Digestion**

|  |  |
| --- | --- |
| Cas9 (CP02/CP01) | 1ug~10ug |
| sgRNA | 800ng~8ug |
| Buffer 3.1 (NEB) | 1ul |
| DNA | 50~100ng |
| ddH2O | To 10ul |

* 37oC incubate for 2 hours (Cas9 seems sensitive to lid heat PCR machine so that we use incubator)
* add 4ug RNase and incubate for 15min at 37oC
* add 1ul STOP buffer and incubate for 15min at 37oC

More details for buffer could be found on the protocol in PNA Bio for Cas9

The concentration of Cas9 and sgRNA depend on the percentage of mtDNA in the library. The more mtDNA in the library, the higher concentration is recommended.