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Protocol: Isolation of genomic DNA with NucleoSpin Blood Kits and PCR pre-check

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Brief description:

This protocol is used to extract gDNA from frozen cell pellet(s) using the NucleoSpin Blood Kits. The manufacturer's protocol recommends different column sizes for cell pellets of various sizes. We strongly encourage users to first test the chosen kit on representative, but non-valuable cells, to ensure the protocol performs well for the cells of interest. gDNA recovery from the column can be estimated using an average value of ~6.6 pg gDNA per cell.

After isolating gDNA, it is highly recommended that you test PCR efficiency of your samples prior to submission by performing a PCR pre-check. This can be done with a very small quantity of (~10 µg) of gDNA from your screen, or, if you don't have sufficient material, with mock cells that are not part of the screen. Ensuring successful PCR prior to submission avoids PCR failures on samples that are already submitted, which can introduce delay in receiving your data, or worse, may result in sample loss.

STEP 1: gDNA extraction

The possible kit sizes are:

- Mini Kit -- for pellets less than 5e6 cells
- L Midi Kit -- for pellets between 5e6 – 2e7 cells
- XL Maxi Kit -- for pellets of 2e7 – 1e8 cells

Note prior to starting: *If using the Mini kit, pellet your cells initially in 200 µL PBS to simplify the protocol.*

Materials and Reagents:

- Machery Nagel NucleoSpin Blood Kit
    - Mini (Takara Cat. #740951)
    - L Midi (Takara Cat. #740954.20)
    - XL Maxi (Takara Cat. #740950)
- PBS
- RNase A (Takara Cat. #740505), lyophilized, dissolve to 20 mg/ml in H2O
- Absolute ethanol
- Extra 1.5 ml, 15 ml, or 50 ml centrifuge tubes (e.g., Falcon)
- A tabletop centrifuge:
    - For the Mini kit: a microcentrifuge capable of 11,000 x g
    - For the L Midi and XL Maxi: swing-out buckets rotor capable of 3,500 g
- Vortex
- Heat block and water bath
- P5 and P7 primers specific to the library vector, PCR materials and reagents

Getting started:

- Prepare the heat block, water bath, and reagents as described in the manufacturer's protocol.
- Equilibrate cell pellets to room temperature (15–25°C).
- For the Mini Kit: spin down cells and resuspend in 200 µL PBS, if not previously done.
- For the XL Maxi Kit: If your cell pellet contains more than the manufacturer's recommended amount, divide the pellet across multiple columns.

Protocol: *Use the manufacturer's instructions with the following modifications*

1. **Lysing cell pellets**

    ➢ Incubate the cell pellet, PBS, Proteinase K and lysis buffer mixture at 70°C overnight in the water bath.

    We have determined that incubating the samples overnight with Proteinase K increases gDNA yields, improves gDNA quality, and increases the likelihood of PCR success.

2. **Precipitating DNA**

    ➢ Remove the lysate from the water bath the following morning, allowing it to cool to room temperature before adding RNase A.

    ➢ Add RNase A (20 mg/ml) and incubate for 5 min at room temperature:
        - Mini: add 1 µL
        - L Midi: add 4.1 µL
        - XL Maxi: add 20 µL

The original NucleoSpin Blood Mini protocol does not call for the addition of RNAse. However, the presence of RNA contamination in gDNA samples can skew quantification when using absorbance-based methods for determining sample concentrations. Furthermore, large amounts of RNA contamination can have a negative impact on subsequent PCR steps. For these reasons we highly recommend using RNase when performing gDNA isolations.

- ➢ When proceeding with the ethanol precipitation, be sure to immediately mix the sample after adding absolute ethanol to avoid high local ethanol concentration.

3. **Binding DNA**

- ➢ Avoid drops on the rim of the column and keep columns in an upright position as liquid may pass through the ventilation slots on the rim of the column even if the caps are closed.

4. **Eluting DNA**

- ➢ Incubate the column with pre-warmed (to 70˚C) elution butter at room temperature for 5 min. An overnight incubation at 4˚C can lead to a modest increase in yield.

- ➢ <u>Optional, but recommended for precious samples:</u> use a PCR inhibitor removal kit on eluted gDNA (Zymo Research, #D6030).

While not necessary, running the gDNA through a PCR inhibitor removal column can reduce the likelihood of PCR failure. There is no sample loss associated with the inhibitor removal columns.

5. **Diluting and storing gDNA**

- ➢ Measure the concentration of gDNA, preferably with Qubit, and, if necessary, dilute to 200 ng/µL with an elution buffer. Calculate the dilutions based on the actual measured volume of eluted DNA, as there is always some loss due to the column.

The Qubit detects dsDNA but does not inform on the presence of RNA. Using a Nanodrop to measure the 260/280 ratio will inform about the total nucleic acid concentration and presence of proteins contaminants, but will not distinguish between RNA and DNA. Qubit and Nanodrop readings are often correlated but NanoDrop readings can be 2 – 3 fold higher than Qubit. We recommend the use of Qubit.

- ➢ Store at 4°C for short term or -20°C for long term.

STEP 2: PCR pre-check

Select and order correct primers:

- ➢ Refer to the bottom of this page to determine which primer pair to use with the appropriate vector from your screen. Amplicon sizes should range from 200-250bp in length.

- ➢ If you have a custom vector and aren't sure which primers to use, please contact GPP-gDNAsubmission@broadinstitute.org for guidance.

- ➢ Order P5 and P7 primers in IDTE pH 8.0 with standard desalting.

- ➢ Dilute P5 primer to 100μM and P7 primer to 5μM in DNase- and RNase-free H_2O.

Primer designs:

P5 Primer Sequences	**NEON**	5'-TCTTGTGGAAAGGACGA-3'
	ARGON	5'-TTGTGGAAAGGACGAAACACCG-'3'
	MAGNESIUM	5'-GCACCGAGTCGGTGCTTTT-'3'
	KRYPTON	5' TCGATTTCTTGGCTTTATATATCTTGTG-3'

P7 Primer Sequences	**KERMIT**	5'-TCTACTATTCTTTCCCCTGCACTGT-3'
	BEAKER	5'-CCAATTCCCACTCCTTTCAAGACCT-'3'
	GONZO	5'-TAAAGCAGCGTATCCACATAGCGT-'3'
	MISS PIGGY	5'-ACCGACTCGGTGCCACTTTTTCAA-3'

Materials and Reagents for PCR:

- Titanium *Taq* DNA Polymerase and PCR buffer (Clontech Takara Cat# 639242)
- dNTPs (Clontech Takara Cat# 4030)
- DMSO (Sigma Aldrich Cat# D9170-5VL)
- PCR plates
- P7 primer
- P5 primer
- gDNA
- Molecular biology grade water
- DNase Away (Thermo Fisher Cat# 7010)
- 70% EtOH

PCR set-up and protocol:

- ➢ Prepare mix inside a PCR hood if available, clean the surface with DNase Away and 70% EtOH. Extreme care should be taken to avoid contamination from / to other DNA preparations.

- ➢ Final contents of each reaction:
  - 10 µL 10x Titanium *Taq* PCR Buffer
  - 8 µL dNTPs
  - 5 µL DMSO
  - 0.5 µL P5 primer 100 µM
  - 10 µg or less of gDNA, but no more than 50 µL by volume
  - 10 µL of P7 primer 5 µM
  - up to 98.5 µL with water
  - finally, 1.5 µL Titanium *Taq* polymerase, 100 µL total volume

1. Make a master mix of water, reaction buffer, dNTP, P5 primer, and finally Titanium *Taq* polymerase. Aliquot into a PCR plate.
2. Add gDNA to each well, reserving at least one well as no-template control by adding water instead.
3. Finally, add the P7 primer.

- ➢ Thermal cycler parameters:

  i. 95 C, 5 minutes
  ii. 95 C 30 seconds (denaturation)
  iii. 53 C 30 seconds (annealing)
  iv. 72 C 20 seconds (extension)

  Back to step 2, total of 28 cycles

  v. 72 C 10 minutes
  vi. 4 C forever

  For gDNA samples, where in the final plating the concentration will be lower than 1ug/well you may want to test increased PCR cycle numbers. We do not recommend going over 35 cycles in order to avoid PCR bias. If you choose to increase PCR cycle number, please make sure to note this in your submission.

- ➢ Run a couple of wells with 10µg gDNA and a couple of No Template Control (NTC) wells.

- ➢ Run PCR products on a gel to confirm the size of the band and spot any contamination.

Expected results:

As an example of what you might see, Figure 1 shows PCR results from three independent CRISPR screen samples (PCR primers: Argon/Kermit; amplicon size: 222bp):

- **Sample A** represents the anticipated results from a properly executed screen.
- **Sample B** represents results from a failed screen. Potential causes of PCR failures include, but are not limited to:
    - High amounts of RNA that co-purified during gDNA isolation
    - Contaminants present in the gDNA sample
    - Modifications to gDNA (e.g., formaldehyde cross-linking)
    - Improper selection during screening, such that the cells did not contain the library

  To avoid PCR failures, we recommend running your gDNA samples through the Zymo purification columns (Zymo Research, #D6030) which will purify samples from PCR inhibitors, as well as checking the DNA concentration with Qubit.

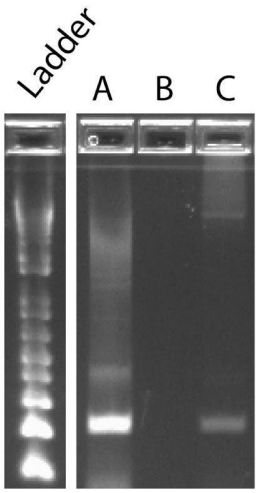

Figure 1. Sample PCR gel from three independent CRISPR screen samples (PCR primers: Argon/Kermit; amplicon size: 222bp).

- **Sample C** demonstrates a screen with low input material. In this case, the PCR product is visible on the gel. While not ideal, it is likely there will be sufficient material for sequencing and deconvolution. You may want to test increased PCR cycle numbers, particularly if you plan to submit gDNA samples below 1ug/well. However, we do not recommend going over 35 cycles to avoid PCR bias.

PCR primer sets:

Perturbation	Vector name	Vector Type	P5 Primer	P7 Primer
CRISPR	pXPR_001	CRISPRko-All-In-One	ARGON	KERMIT
CRISPR	pXPR_003	CRISPRko-GuideOnly	ARGON	KERMIT
CRISPR	pXPR_004	CRISPRko-All-In-One	ARGON	KERMIT
CRISPR	pXPR_005	CRISPRko-All-In-One	ARGON	KERMIT

CRISPR	pXPR_006	CRISPRko-All-In-One	ARGON	KERMIT
CRISPR	pXPR_016	CRISPRko-GuideOnly	ARGON	KERMIT
CRISPR	pXPR_023	CRISPRko-All-In-One	ARGON	BEAKER
CRISPR	pXPR_024	CRISPRko-GuideOnly	ARGON	KERMIT
CRISPR	pXPR_025	CRISPRko-All-In-One	ARGON	KERMIT
CRISPR	pXPR_027	CRISPRko-All-In-One	ARGON	KERMIT
CRISPR	pXPR_028	CRISPRko-All-In-One	ARGON	KERMIT
CRISPR	pXPR_034	CRISPRalt	ARGON	BEAKER
CRISPR	pXPR_036	CRISPRko-GuideOnly	ARGON	KERMIT
CRISPR	pXPR_037	CRISPRko-GuideOnly	ARGON	KERMIT
CRISPR	pXPR_043	CRISPRko-GuideOnly	ARGON	KERMIT
CRISPR	pXPR_044	CRISPRko-All-In-One	ARGON	BEAKER
CRISPR	pXPR_045	CRISPRko-GuideOnly	ARGON	KERMIT
CRISPR	pXPR_048	CRISPRko-All-In-One	ARGON	BEAKER
CRISPR	pXPR_049	CRISPRko-GuideOnly	ARGON	KERMIT
CRISPR	pXPR_050	CRISPRko-GuideOnly	ARGON	KERMIT
CRISPR	pXPR_051	CRISPRko-All-In-One	ARGON	BEAKER
CRISPR	pXPR_052	CRISPRko-GuideOnly	ARGON	
CRISPR	pXPR_053	CRISPRko-GuideOnly	ARGON	KERMIT
CRISPR	pXPR_055	CRISPRko-GuideOnly	ARGON	KERMIT
CRISPR	pXPR_206	CRISPRalt	ARGON	BEAKER
CRISPR	pXPR_212	CRISPRalt	ARGON	BEAKER
CRISPR	pXPR_501	CRISPRa	ARGON	KERMIT
CRISPR	pXPR_502	CRISPRa	ARGON	KERMIT
CRISPR	pRDA_026_027	CRISPRalt	ARGON	BEAKER
CRISPR	pRDA_052	CRISPRalt	ARGON	KERMIT
CRISPR	pRDA_118	CRISPR_GUIDE_ONLY	ARGON	KERMIT
CRISPR	pRDA_186	CRISPR_GUIDE_ONLY	ARGON	KERMIT
CRISPR	pRDA_199	CRISPR_ALL_IN_ONE	ARGON	BEAKER
ORF	pLX_317	ORF-Constitutive	NEON	GONZO
shRNA	pLKO.1	shRNA-constitutive	NEON	KERMIT
shRNA	pLKO_TRC005	shRNA-constitutive	NEON	KERMIT
shRNA	pLKO_TRC006	shRNA-constitutive	NEON	KERMIT
shRNA	pLKO_TRC008	shRNA-constitutive	NEON	KERMIT
shRNA	pLKO_TRC009	shRNA-constitutive	NEON	KERMIT
shRNA	pLKO_TRC016	shRNA-constitutive	NEON	KERMIT
shRNA	pLKO_TRC017	shRNA-constitutive	NEON	KERMIT
shRNA	pLKO_TRC018	shRNA-constitutive	NEON	KERMIT

shRNA	pLKO_TRC019	shRNA-constitutive	NEON	KERMIT
shRNA	pLKO_TRC020	shRNA-constitutive	NEON	KERMIT
shRNA	pLKO_TRC021	shRNA-constitutive	NEON	KERMIT
shRNA	pLKO_TRC022	shRNA-constitutive	NEON	KERMIT
shRNA	pLKO_TRC023	shRNA-constitutive	NEON	KERMIT
shRNA	pLKO_TRC024	shRNA-constitutive	NEON	KERMIT
shRNA	pLKO_TRC039	shRNA-constitutive	NEON	KERMIT
shRNA	pLKO_TRC040	shRNA-constitutive	NEON	KERMIT
shRNA	pLKO_TRC044	shRNA-constitutive	NEON	KERMIT
shRNA	pLKO_TRC046	shRNA-constitutive	NEON	KERMIT
shRNA	pLKO_TRC047	shRNA-constitutive	NEON	KERMIT
shRNA	pLKO_TRC060	shRNA-constitutive	NEON	KERMIT
shRNA	pLI_TRC912	shRNA-inducible	NEON	KERMIT
shRNA	pLI_TRC913	shRNA-inducible	NEON	KERMIT
shRNA	pLI_TRC914	shRNA-inducible	NEON	KERMIT
shRNA	pLI_TRC931	shRNA-inducible	NEON	KERMIT
shRNA	pLI_TRC950	shRNA-inducible		KERMIT