# Protocols for scDASH

**Preparation of sgRNA**

1. PCR amplification of DNA template of sgRNA
	1. Order IVT primers that generate the DNA template when annealed:
		1. sgRNA specific forward primer with T7 promoter & 14-nt overlap sequence (54-55 nt):
			* TTCTAATACGACTCACTATA(G)(sgRNA-20)GTTTTAGAGCTAGA
				+ G is added upstream if sgRNA sequence does not begin with a G, in order to satisfy the sequence requirements of T7 RNA polymerase promoter
		2. SpCas9 common reverse primer containing the sequence of the sgRNA scaffold (80 nt):
			* AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC
	2. Perform PCR with a high fidelity (with proofreading function) polymerase, in our case KAPA HiFiTM HotStart DNA Polymerase was used.
	3. PCR Clean-up - NucleoSpin® Gel and PCR Clean-up was used in our case.
	4. Measure DNA concentration.
	5. Verify amplicon size by agarose gel electrophoresis.
2. *In vitro* transcription
	1. HiScribe™ T7 Quick High Yield RNA Synthesis Kit (NEB) was used.
		1. 1 picomole (about 75 ng of a 120 bp PCR product) can be used in a 30 μl *in vitro* transcription reaction. Protocol can be found here:

|  |  |
| --- | --- |
| **Component** | **Volume**  |
| NTP Buffer Mix | 10 μl |
| Template DNA | X μl (75 ng)  |
| T7 RNA Polymerase Mix | 2 μl |
| Nuclease-free water | to 30 μl |

* + 1. Incubate at 37°C overnight in a thermocycler.
		2. Purify the transcripts – Agencourt AMPure XP was used.
		3. Verify transcript size using Fragment Analyzer Automated CE System (DNF 472).

***In vitro* Cas9 Digestion**

1. Formation of ribonucleoprotein (RNP) complex

|  |  |
| --- | --- |
| **Component** | **Volume**  |
| 2X Cas9 Buffer | 5 μl |
| sgRNA | X μl (2700 ng)  |
| 1 µM (159 ng/μl) SpCas9 Nuclease (NEB) | 0.57 μl (90 ng) |
| Nuclease-free water | to 10 μl |

* To calculate the input ratio of Cas9 and sgRNA to sample nucleic acid, it is assumed that 90% of each cDNA sample is comprised of the rRNA substrate of our target
	+ In a 1 ng sample, rRNA substrate makes up 0.9 ng
* To assure the most thorough Cas9 activity possible, and given that Cas9 is a single-turnover enzyme *in vitro*, 100-fold excess of Cas9 protein and a 3000-fold excess of sgRNA relative to the target will be used
	+ With 0.9 ng of rRNA cDNA substrate, 90 ng Cas9 and 2700 ng sgRNA will be used
1. Incubate reaction at 37°C for 10 min.
2. CRISPR/Cas9 Digestion
	1. Add 1 ng library to the 10 μl reaction from previous step.
3. Add another volume (5 μl) of 2X Cas9 Buffer.
4. Top up with nuclease-free water to a total volume of 20 μl.
5. Incubate at 37°C for 2 hr and 30 min.
6. Cas9 Removal
	1. Add 1 μl (at >600 mAU/mL) of Proteinase K (Qiagen, Hilden, Germany).
	2. Incubate at 37°C for 15 min.
7. Purification of digested library - Agencourt AMPure XP was used.

**PCR Enrichment of Non-targeted Sequences**

* Reaction Setup:

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| --- | --- |
| **Component** | **Volume**  |
| 5X KAPA Buffer | 3 μl |
| 5X TAPs Buffer | 1 μl  |
| 40% PEG4000 | 1 μl  |
| 10 mM dNTPs | 0.45 μl  |
| Template DNA | 1.5 μl |
| KAPA HiFi HotStart Polymerase | 0.3 μl |
| 5 μM P5 primer | 1.25 μl |
| 5 μM P7 primer | 1.25 μl |
| Nuclease-free water | 6.5 μl |

* Thermocycling programme:

|  |  |  |  |
| --- | --- | --- | --- |
| **Step** | **Temperature** | **Duration** | **Cycles** |
| Initial denaturation | 95°C | 3 min | 1 |
| Denaturation  | 98°C | 20 s | 20 |
| Annealing | 60°C | 15 s |
| Extension | 72°C | 30 s |
| Final extension | 72°C | 2 min | 1 |

**Depletion Efficiency Characterisation by Quantitative PCR**

* Reaction Setup:

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| --- | --- |
| **Component** | **Volume**  |
| 2X SYBR Green Mix (Roche) | 5 μl |
| Template DNA | X μl (0.1 ng)  |
| 10 µM primer mix | 0.5 μl  |
| Nuclease-free water | to 10 μl |

* Thermocycling programme:

|  |  |  |  |
| --- | --- | --- | --- |
| **Step** | **Temperature** | **Duration** | **Cycles** |
| Initial denaturation | 95°C | 5 min | 1 |
| Denaturation  | 95°C | 10 s | 40 |
| Annealing | 58°C | 15 s |
| Extension | 72°C | 10 s |

# Recipe

**2X Cas9 Buffer**

|  |  |  |
| --- | --- | --- |
| **Component** | **Volume (or Mass)** | **Final concentration** |
| 1 M Tris (pH 8.0) | 200 μl | 100 mM |
| 5 M NaCl | 80 μl | 200 mM |
| 1 M MgCl2 | 40 μl | 20 mM |
| TCEP (powder) | 1.1466 mg | 2 mM |
| Nuclease-free water | to 2 mL | / |