SISPA

cDNA first strand synthesis

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| **Primer** | **Sequence (5’ > 3’)** |
| *Sol-A primer* | GTT TCC CAC TGG AGG ATA NNN NNN NNN |
| *Sol-B primer* | |  |  | | --- | --- | |  | GTT TCC CAC TGG AGG ATA | |

Combine initial components in PCR reaction tube, mix by pipetting and briefly centrifuge components.

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| Component | Volume |
| *40pmol* Sol-A Primer | 1μl |
| 10mM dNTP Mix (NEB N0447L) | 1μl |
| Sample RNA (10pg-5μg total RNA or 10pg-500ng mRNA) | Up to 11μl (5μl suggested) |
| Nuclease free water (ThermoFisher 4387936) | Make up to 13μl total volume |

1. Heat the RNA-Primer Mix at 65℃ for 5 minutes, then incubate on ice for at least 1 minute.

Prepare RT reaction mix

1. Vortex then briefly centrifuge then 5xSSIV Buffer
2. Combine the following components in a fresh reaction tube.

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| Component | Volume |
| 5x SSIV Buffer (ThermoFisher 18090200) | 4μl |
| 100mM DTT | 1μl |
| Ribonuclease Inhibitor (Not essential, make up with H2O if not using) (RNaseOut Life Technologies 10777019) | 1μl |
| SuperScriptTM IV Reverse Transcriptase (200 U/μl) (ThermoFisher 18090200) | 1μl |

1. Cap the tube, mix then briefly centrifuge the contents.

Combine and incubate for first strand cDNA

1. Combine RT reaction mix and annealed RNA mix for a total reaction mix of 20μl.
2. Incubate at 23℃ for 10 minutes, followed by an incubation at 50-55℃ for 10 minutes.
3. Inactivate the reaction by incubating at 80℃ for 10 minutes.
4. Use RT reaction immediately or store at -20℃.

cDNA second strand synthesis

1. 5ul Sequenase mix 1 was added to cDNA reaction and incubated at 37C for 8minutes.

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| Component | Volume |
| 5X sequenase buffer (ThermoFisher 0775Z1000UN) | 1ul |
| ddH2O | 3.75ul |
| Sequenase enzyme (ThermoFisher 0775Z1000UN) | 0.15ul |

1. Sequenase mix 2 was added and incubated at 37C for 8 minutes

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| --- | --- |
| Component | Volume |
| Sequenase dilution buffer | 0.45ul |
| Sequenase enzyme | 0.15ul |

Purification using Agencourt AMPure beads – either clean or dilute cDNA before entering PCR

According to AMPure XP bead protocol

1. Resuspend beads stock by vortexing.
2. Transfer sample to DNA LoBind Eppendorf.
3. Add 1.8μl AMPure XP per 1μl sample, mix by pipetting and incubate samples at RT for 5 minutes to allow binding.
4. Place beads against magnet, allow to pellet (~2mins)
5. Remove supernatant leaving ~5μl behind to ensure beads don’t detach from magnet.
6. Add 200μl 70% Ethanol to each tube then incubate at RT for 30 seconds.

N.B. Keep tube by magnet whilst washing with ethanol to maintain bead pellet

1. Aspirate ethanol – beads do not detach as easily with ethanol so can take whole supernatant.
2. Repeat step 6-7.
3. Allow pellet to dry for 30 seconds before removing from magnet.
4. Add 40μl elution buffer, mix by pipetting then incubate at RT for 2 mins.
5. Place beads against magnet, allow beads to pellet (~2mins).
6. Elute purified DNA fragments from beads before transferring to new tube.

*Optional quantification of un-amplified cDNA by Qubit or Nanodrop.*

Sequence Independent Single Primer Amplification – do duplicates or triplicates to improve coverage

1. Combine the following reaction mixture to a final volume of 25μl.

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| Component | Volume |
| Sample cDNA | 12ul |
| 2x Q5 Master Mix (NEB M0492L) | 12.5μl |
| 100pmol/ul Sol-B Primer | 0.5ul |

1. PCR cycling as follows: 98 °C for 30 s, followed by 25 cycles of 98 °C for 10 s, 54 °C for 30 s, and 72 °C for 1 min, with a final extension at 72 °C for 10 min.

Purification using Agencourt AMPure beads

According to AMPure XP bead protocol

1. Resuspend beads stock by vortexing.
2. Transfer sample to DNA LoBind Eppendorf.
3. Add 1.8μl AMPure XP per 1μl sample, mix by pipetting and incubate samples at RT for 5 minutes to allow binding.
4. Spin down then place beads against magnet, allow to pellet (~2mins)
5. Remove supernatant leaving ~5μl behind to ensure beads don’t detach from magnet.
6. Add 200μl 70% Ethanol to each tube then incubate at RT for 30 seconds.

N.B. Keep tube by magnet whilst washing with ethanol to maintain bead pellet

1. Aspirate ethanol – beads do not detach as easily with ethanol so can take whole supernatant.
2. Repeat step 6-7.
3. Spin down then place back on magnet and remove any extra ethanol.
4. Allow pellet to dry for 30 seconds before removing from magnet.
5. Add 40μl n.f.H2O, mix by pipetting then incubate at RT for 2 mins.
6. Spin down then place beads against magnet, allow beads to pellet (~2mins).
7. Elute purified DNA fragments from beads before transferring to new tube.

*Optional quantification of amplified cDNA by Qubit or Nanodrop.*