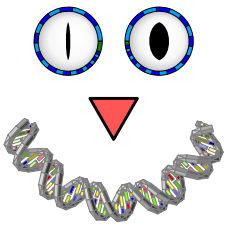
Sample Preparation - PCR cDNA Barcoding

GrinGene Bioinformatics



Version: 1.1.6
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Whakapapa / Tohutoro (References)

- Based on the ONT Community Protocols for PCR-cDNA Barcoding (SQK-PCB109) [PCB_9092_v109_revA_10Oct2019, MinION / GridION / PromethION (https://community.nanoporetech.com/protocols/pcr-cdna-barcoding_sqk-pcb109/v/PCB_9092_v109_revA_10Oct2019)]
- Updated based on the ONT Community Protocols for PCR-cDNA Barcoding (SQK-PCB111) [PCR-cDNA Barcoding Kit (SQK-PCB111.24) (https://community.nanoporetech.com/docs/prepare/library_prep_protocols/pcr-cdna-barcoding-kit-sqk-pcb111-24/v/pcb_9155_v111_revh_18may2022?devices=minion)]
- And the Ligation Sequencing V14 - Direct cDNA Sequencing (SQK-LSK114) (https://community.nanoporetech.com/docs/prepare/library_prep_protocols/ligation-sequencing-v14-direct-cdna-sequencing/v/dcs_9187_v114_revc_19apr2023) protocol
- And the cDNA-PCR sequencing - sequence-specific (SQK-PCS111) (https://community.nanoporetech.com/docs/prepare/library_prep_protocols/cdna-pcr-sequencing-sequence-specific-sqk-pcs111/v/cpss_9185_v111_reva_19apr2023) protocol
- And the Rapid sequencing DNA V14 - barcoding (https://community.nanoporetech.com/docs/prepare/library_prep_protocols/rapid-sequencing-gdna-barcoding-sqk-rbk114/v/rbk_9176_v114_revj_27nov2022) protocol
- Additional explanations by David Eccles (knowledge from ONT talks, and practical experience)
- Additional preparation optimisations by Sventja von Daake (practical experience)

Scope and Purpose

This procedure is for preparing an RNA library for sequencing on a ONT R10.4.1 MinION flow cell, where high sequence accuracy and low crossover of barcodes is desired. Yield is reduced compared to a ligation-based sequencing protocol, but sample-prep based contamination is minimal (about 0.1%) due to the rapid attachment chemistry. It is recommended that a Flow Cell QC (flow-cell-QC.html) is carried out just prior to doing sample preparation, to make sure that a working Flow Cell will be available for a freshly-prepared sample, and to ensure that the protective environment used for shipping the flow cells is maintained for as long as possible.

The cDNA synthesis steps add ONT anchor sequences onto the template sequences. These allow the ONT universal rapid barcode primers to bind and extend. The strand-switch primer includes an additional unique molecular identifier (UMI; represented by 'V' in the SSP sequence below) to help with read deduplication during post-sequencing QC, and also includes 3 2' O-methyl RNA Gs to anchor on to the end of sequences that have been created using a reverse polymerase:

```
SSP 5' - TT-TCTGTTGGTGCTGATATTGC-TTTVVVVTTVVVVTTVVVVTTVVVVTTT-mGmGmG - 3'
VNP 5' - AC-TTGCCTGTCGCTCTATCTTC-TTTTTTTTTTTTTTTTTTTTVN - 3'
```

[note: the initial TT/AC were present in earlier barcode primers, but not the primers in the newest V14 kits]

The `TTT...TTTVN` region of the VNP can be replaced by gene-specific sequences if targeted amplification is desired, bearing in mind that the reverse-transcription reaction happens at 42ºC.

Barcodes are added via PCR, extending ONT primers. The bottom and top primers have different flanking sequences, allowing for strand-specific sequence identification:

```
BOT 5' - * - ATCGCCTACCGTGA - barcode - TCTGTTGGTGCTGATATTGC - 3'
TOP 5' - * - ATCGCCTACCGTGA - barcode - TTGCCTGTCGCTCTATCTTC - 3'

*: proprietary adapter bonding site
```

Note that because this universal primer anchor sequence (at the 3' end) is in the same orientation as the SSP and VNP primers, it will not bind directly to them, but will bind to their reverse-complement partner (as found on a fully-extended PCR product). This will require an additional cycle of PCR in order for the joining to happen.

Finally, the Kit14 Rapid Adapter is added, bonding with the ONT primers [note: this is the 'RAP T' sequence from ONT documentation; whether this sequence is different for Kit 14 RA is unclear]:

```
5' - TTTTTTTTCCTGTACTTCGTTCAGTTACGTATTGCT - * - 3'

*: proprietary adapter bonding site
```

[See barcode-sequences (barcode-sequences.html) for the barcode sequences]

Potential Hazards

The most dangerous component of the PCR cDNA Barcoding Kit is glycerol (CAS#: 56-81-5; EC#: 200-289-5), which is not classified as hazardous. All kit components are in small quantities and are unlikely to be considered a significant hazard, as long as standard laboratory practises are observed. For more details, please refer to the PCR cDNA Barcoding Kit Safety Data Sheet (https://assets.ctfassets.net/obrbum38fjyj/3PKpJSYCYnwG6OG3aP08m4/6fb32f5ba04b3840ffda0c33fd3c9304/SQK-PCB111.24-English_.pdf).

Safety / Procedural Precautions

- Ultra-long DNA is sensitive to breakage. While the fragmentation and PCR barcoding process used in this protocol will break DNA down to lengths where shearing is not an issue, it is good practise to get into the habit of carefully handling samples containing *any* DNA. In particular, take extra care to mix only via the prescribed method (i.e. by pipetting, vortexing, or flicking).

Before You Begin

RNA should be eluted / extracted in TE buffer, or in an RNA storage buffer. RNA is best converted to double-stranded cDNA as soon as possible after extraction. If storage is necessary, extracted RNA is best stored in a non-cycling 4°C fridge for up to a month, or in a -70°C freezer if stored for longer periods of time (see here (https://community.nanoporetech.com/knowledge/know-how/rna-stability) and here (https://community.nanoporetech.com/knowledge/know-how/library-stability)). Freeze-thaw cycles should be avoided or minimised, as the freeze-thaw cycle is a large contributor to RNA degradation.

Materials and Equipment

Materials (ONT)

Reverse Transcription

- PCR cDNA Barcoding Kit (SQK-PCB111); thaw all reagents at RT if frozen, then mix **by pipetting**, spin down and store on ice:
    - Strand-switching Primer II (SSPII), at least 2 µl per sample

PCR

- PCR cDNA Barcoding Kit (SQK-PCB111); thaw all reagents at RT if frozen, then mix, spin down and store on ice:
    - Barcode Primers, at least 1.5 µl per sample
    - Elution Buffer (EB), at least 12 µl per sample

Adapter Binding

- Rapid Adapter Auxiliary V14 (EXP-RAA114, or another rapid kit)
    - Rapid Adapter (RA), at least 1.5 µl per run
    - Adapter Buffer (ADB), at least 3.5 µl per run

Equipment & Consumables (Other Suppliers)

- P1000 pipette & tips
- P200 pipette & tips
- P100 pipette & tips
- P20 pipette & tips
- P10 pipette & tips
- P2 pipette & tips
- Timer
- Thermal cycler
- Gentle agitator (e.g Hula mixer or rotator mixer, or thermomixer)
- Magnetic rack for 1.5µl tubes
- Microfuge
- Vortex mixer
- Pre-chilled freezer block at -20°C for 200µl tubes (e.g. Eppendorf 022510509)
- Fluorometer (e.g. Quantus)

Reverse Transcription

- polyTVN Reverse Transcription primer (e.g. VNP from old PCB109 kit) [at least 1µl per sample]
    - 5' /5phos/ACTTGCCTGTCGCTCTATCTTCTTTTTTTTTTTTTTTTTTTTVN 3'
    - HPLC purified
    - /5phos/ is optional (used for direct cDNA sequencing with the ligation kit)
    - diluted / reconstituted to 2µM
- 1.5 ml Eppendorf DNA LoBind tubes [at least 1 per sample]
- 0.2 ml thin-walled PCR tubes [at least 2 per sample]
- Agencourt AMPure XP beads (or RNase-free XP beads) [at least 36 µl per sample]
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937) [at least 12 µl per sample]

- 10 mM dNTP solution (e.g. NEB N0447) [at least 1 µl per sample]
- Maxima H Minus Reverse Transcriptase (200 U/µl) with 5x RT Buffer (ThermoFisher cat # EP0751), or SuperScript IV Reverse Transcriptase (ThermoFisher cat # 18090010 (https://www.thermofisher.com/order/catalog/product/18090010)) [at least 1 µl per sample]
- RNaseOUT, 40 U/µl (Life Technologies, cat # 10777019) [at least 1 µl per sample]
- 4 ng polyA+ RNA per sample (or 200 ng total RNA)

PCR

- 1.5 ml Eppendorf DNA LoBind tubes [at least 2 per sample]
- 0.2 ml thin-walled PCR tubes [at least 2 per sample]
- Nuclease-free water (e.g. ThermoFisher, cat # AM9937) [at least 7 µl per sample]
- Agencourt AMPure XP beads [at least 30 µl per sample]
- LongAmp Hot Start Taq 2X Master Mix (e.g. NEB M0533S) [at least 50 µl per sample]; thaw at RT and mix **by pipetting** until bits go away, store on ice
- Exonuclease I (e.g. NEB M0293) [at least 1 µl per sample]; mix **by pipetting**, store on ice
- Freshly prepared 80% ethanol in nuclease-free water [at least 200 µl per sample]

Input QC

Check to make sure that there is sufficient extracted RNA for amplification (at least 1 ng PolyA+ RNA, or ~50 ng total RNA). An ideal situation would be to use, as input, RNA with a concentration greater than 20ng/µl, so that it can be assessed for quality on a Nanodrop:

- 260/280 ratio ~ 2.0 (lower suggests DNA, protein, or phenol contamination)
- 260/230 ratio ~2.0-2.2 (lower suggests a contaminated sample)

RNA should also be assessed for quality on a TapeStation or electrophoresis gel prior to sample preparation (especially for new extraction protocols or sample types) to check for degradation, verifying that there is sufficient long RNA over 1 kb – when using a TapeStation, RNA that produces good sequencing runs has an RNA integrity number (RIN) of 7 or higher.

Method – Reverse Transcription

Reverse transcription master mixes

1. Into a 0.2 ml PCR tube, add the following as a primer master mix:
    1. 1 µl polyTVN primer (VNP) per sample + 0.2 µl extra (e.g. 6.2 µl for 6 samples)
    2. 1 µl dNTPs (10 mM) per sample + 0.2 µl extra (e.g. 6.2 µl for 6 samples)
2. Into another 0.2 ml PCR tube, add the following as a buffer master mix:
    1. 4 µl Maxima H Minus 5x RT Buffer per sample + 0.8 µl extra (e.g. 24.8 µl for 6 samples)
    2. 1 µl RNaseOUT per sample + 0.2 µl extra (e.g. 6.2 µl for 6 samples)
    3. 1 µl Nuclease-free water per sample + 0.2 µl extra (e.g. 6.2 µl for 6 samples)
    4. 2 µl Strand Switching Primer II (SSPII) per sample + 0.4 µl extra (e.g. 12.4 µl for 6 samples)

Reverse transcription and second-strand synthesis (carried out for each sample)

1. Transfer 4 ng PolyA+ RNA (or 200 ng total RNA) into a 0.2 ml PCR tube on a pre-chilled freezer block. [Note: total RNA concentration should be at least 20 ng/µl]
2. Adjust the volume to 9 µl with nuclease-free water
3. Add 2 µl primer master mix (prepared in the previous steps) to each sample tube (to a total volume of 11 µl)
4. Mix gently **by flicking** to avoid unwanted shearing
5. Spin the samples down briefly (1-3s) in a microfuge
6. Incubate for 5 minutes at 65°C to anneal the primers 1 Snap cool for 1 minute on the freezer block
7. Add 8 µl buffer master mix (prepared in the previous steps) to each sample tube (to a total volume of 19 µl)
8. Mix gently **by flicking** to avoid unwanted shearing
9. Spin the samples down briefly (1-3s) in a microfuge
10. Incubate all sample tubes in a thermal cycler for 2 minutes at 42°C [DE – would be worth checking if this step is necessary]
11. Add 1 µl of Maxima H Minus Reverse Transcriptase to each sample tube (to a total volume of 20µl)
12. Mix gently **by flicking** to avoid unwanted shearing
13. Spin the samples down briefly (1-3s) in a microfuge
14. Carry out first and second-strand synthesis for all samples in a thermal cycler using the following protocol:

Cycle Step	Temperature	Time
Reverse transcription and strand switching	42°C	90 mins
Heat inactivation	85°C	5 mins
Hold	4°C	∞

This is a good stopping point for sample storage (e.g. overnight at 4°C), as the RNA has now been converted into more stable cDNA.

Method – PCR Barcoding and DNA Amplification

Note: if preparing a library for fewer than four samples, carry out multiple PCR reactions on 5 µl from each sample so that the total amplified volume is at least 20 µl. This ensures that there is enough PCR product generated for optimal flow cell performance.

PCR Master Mix

1. Into a 1.5 ml LoBind tube, add the following as a PCR master mix:
    1. 6.75 µl Nuclease-free water per reaction + 0.25 µl extra (e.g. 40.75 µl for 6 reactions)
    2. 12.5 µl 2x LongAmp Hot Start Taq Master Mix per reaction + 0.45 µl extra (e.g. 75.45 µl for 6 reactions)

PCR barcoding and amplification (carried out for each reaction)

1. Transfer 5 µl reverse-transcribed sample into a 0.2 ml PCR tube on a pre-chilled freezer block
2. Add 0.75 µl Unique Barcode Primer (BP01-24) to the respective sample tube. If a sample is being split into multiple reactions, the same barcode primer can be used for each reaction (if desired).
3. Add 19.25 µl PCR master mix (prepared in the previous steps) to each sample tube (to a total volume of 25 µl)
4. Mix gently **by pipetting**
5. Amplify all tubes together in a thermal cycler using the following cycling conditions (14 cycles amplification):

Cycle Step	Temperature	Time
Initial denaturation	95°C	30 secs
14 cycles* of…		
/ Denaturation	95°C	15 secs
Annealing	62°C	15 secs
\ Extension	65°C	2 mins
Final extension	65°C	6 mins
Hold	4°C	–

Additional notes

- * 14 cycles is recommended as a starting point. However, the number of cycles can be adjusted between the values shown based on sample experience (with a target output concentration of at least 20 ng / µl). For further information, please read The effect of varying the number of PCR cycles in the PCR-cDNA Sequencing Kit (https://community.nanoporetech.com/knowledge/know-how/varying-pcr-cycles) document.
- For longer amplified cDNA lengths, the extension time can be adjusted (1 minute per kb)
- *TODO: check different annealing temperatures. The Rapid PCR Barcoding Kit uses an annealing temperature of 56°C (for what should be the same primer set).*

Adapter Digestion (carried out for each reaction)

[TODO: to reduce tube transfers, could this be done in 1.5ml tubes instead?]

1. Spin the tubes down briefly (1–3s) in a microfuge to settle condensate
2. Add 1 µl Exonuclease 1 (20 units) directly to each PCR tube
3. mix **by pipetting**
4. Spin the tubes down briefly (1–3s) in a microfuge
5. Carry out free adapter digestion for all samples in a thermal cycler using the following protocol:

Cycle Step	Temperature	Time
Exonuclease digest	37°C	15 mins
Heat inactivation	80°C	15 mins
Hold	4°C	∞

DNA Concentration (carried out for each reaction)

1. Resuspend the AMPure XP beads **by vortexing**
2. Prepare 400 µl of fresh 80% ethanol per reaction in nuclease-free water (e.g. 2400 µl for 6 reactions)
3. Transfer the amplified DNA to a clean labelled 1.5 ml Eppendorf DNA LoBind tube
4. Add 21 µl of resuspended AMPure XP beads (i.e. 0.8X) to the reaction and mix **by pipetting**
5. Incubate on a gentle agitator (e.g Hula mixer or rotator mixer, or thermomixer) for at least 5 minutes at RT (18–23°C).
6. Spin the tubes down briefly (1–3s) in a microfuge
7. Pellet the beads in a magnetic rack until the eluate is clear and colourless.
8. Keeping the tube on the magnet, pipette off and discard the supernatant
9. Keeping the tube on the magnet, slowly add 200 µl of 80% ethanol to cover (but not disturb) the pellet
10. Keeping the tube on the magnet, pipette off and discard the ethanol and collected contaminants, leaving the pellet behind
11. Keeping the tube on the magnet, slowly add another 200 µl of 80% ethanol to cover (but not disturb) the pellet
12. Keeping the tube on the magnet, pipette off and discard the ethanol and collected contaminants, leaving the pellet behind
13. Spin the tubes down briefly (1–3s) in a microfuge
14. Place the tubes back on the magnet and pipette off any residual ethanol
15. Allow to air dry for at most 30 seconds, but do not dry the pellet to the point of cracking. This will happen shortly after the pellet loses shine from the liquid
16. Add 12 µl of Elution Buffer (EB)
17. Remove the tube from the magnetic rack, and resuspend **by flicking**
18. Spin the tubes down briefly in a microfuge
19. Incubate on a gentle agitator (e.g Hula mixer or rotator mixer, or thermomixer) for at least 10 minutes at RT (18–23°C)
20. Pellet the beads in a magnetic rack until the eluate is clear and colourless
21. Quantify 1 µl of eluted sample using a fluorometer. The DNA concentration should be at least 20ng/µl

This is a good stopping point; concentrated cDNA can be stored on beads in a non-cycling fridge at

4°C. Consider running 1µl of each sample on a gel (or a TapeStation / Bioanalyzer) to determine the average cDNA / transcript length

Barcode pooling

1. If beads have been stored, resuspend **by flicking**
2. Pellet the beads in a magnetic rack until the eluate is clear and colourless
3. Add equimolar quantities of each sample (at least 1µl per sample) to a clean labelled 1.5 ml Eppendorf DNA LoBind tube
4. Transfer 25 fmol of this mix into another clean labelled 1.5 ml Eppendorf DNA LoBind tube as a barcoded DNA library.
5. Make the volume up to 11µl in Elution Buffer (EB)

Additional notes

- Molarity is based on average fragment length. Assuming 1.5kb average length (a reasonable assumption for cDNA), the fmol amount is approximately equal to the ng amount (e.g. 25 ng = 25 fmol).
- The sequencing adapter used for R10.4.1 flow cells has a higher capture rate, enabling lower flow cell loading amounts to give optimal pore occupancy.
- The 25 fmol amount is based on the LSK114 protocol, which recommends 10–20 fmol (accounting for a small amount of non-adapted reads). A much higher amount is suggested in the rapid barcoding protocol (800 ng, or 80 ~ 800 fmol).
- If the quantity of amplified cDNA is above 25 fmol, the remaining cDNA can be stored at 4°C for up to a month for another sequencing experiment. If storing for longer, cDNA (eluted from magnetic beads if necessary) can be stored at −70°C. Avoid multiple freeze–thaw cycles to prevent DNA degredation.

Method – Adapter addition

Adapter preparation

1. Thaw the adapter buffer at RT, mix **by vortexing**, then spin down briefly (1–3s) in a microfuge, and place on ice
2. Mix the Rapid Adapter (RA) **by pipetting**, then spin down briefly (1–3s) in a microfuge, and place on ice
3. Into a 0.2 ml PCR tube, add the following as diluted rapid adapter:
    1. 1.5 µl Rapid Adapter (RA)
    2. 3.5 µl Adapter Buffer (ADB)
4. Add 1µl of diluted rapid adapter to the barcoded DNA library
5. Mix gently **by flicking** to avoid unwanted shearing
6. Spin the library down briefly (1–3s) in a microfuge
7. Incubate for at least 5 minutes at RT (18–23°C)

Note: The rapid adapter is diluted into a total volume of 5µl, of which only 1µl is used for sequencing. Left over diluted adapter can be stored in a -20°C freezer for use in additional rapid sequencing experiments. The lifetime of the diluted rapid adapter is not yet known (it lasts at least a week), so it is recommended to label the tube with the date of dilution to help with establishing lifetimes in the future.

Method – Flow Cell Loading

The sequencing library has now been prepared for sequencing. Proceed with the 12µl of library as input for ONT R10.4.1 flow cell loading, as per the RBK114.24 kit:

- Flongle Flow Cell (https://community.nanoporetech.com/docs/prepare/library_prep_protocols/rapid-sequencing-gdna-barcoding-sqk-rbk114/v/rbk_9176_v114_revj_27nov2022/loading-the-flongle-flow-cell?devices=flongle)
- MinION Flow Cell (https://community.nanoporetech.com/docs/prepare/library_prep_protocols/rapid-sequencing-gdna-barcoding-sqk-rbk114/v/rbk_9176_v114_revj_27nov2022/priming-and-loading-the-spoton-flow-cell?devices=minion)
- PromethION Flow Cell (https://community.nanoporetech.com/docs/prepare/library_prep_protocols/rapid-sequencing-gdna-barcoding-sqk-rbk114/v/rbk_9176_v114_revj_27nov2022/priming-and-loading-the-promethion-flow-cell?devices=promethion)

A quick summary of loading for each type of flow cell follows; please consult the protocols above for more information.

Loading - Flongle Flow Cells

This uses the gentle negative-pressure loading method from the community, with 5µl of library loaded in 30µl mix:

https://community.nanoporetech.com/posts/a-very-gentle-relatively (https://community.nanoporetech.com/posts/a-very-gentle-relatively)

1. Prepare Flow Cell Flush solution:
    - 117 µl Flow Cell Flush (FCF)
    - 3 µl Flow Cell Tether (FCT)
    - mix ***by pipetting***
2. Unseal the flow cell
3. Add tape to the rectangular waste port (see the sticker)
4. Drop 30 µl of flush solution onto the loading port to form a dome
5. Place pipette into loading port and dial up about 5 µl to check for bubbles (dial up to remove bubbles if they exist)
6. If liquid is not dropping from the loading port, place pipette into the exposed waste port and dial up 30 µl or until liquid starts dropping from the loading port
7. Set pipette to 30 µl, press down while in mid air to expel air, then place into the exposed waste port and slowly release the plunger. If liquid starts dropping from the loading port, stop releasing and lift up the pipette
8. Repeat the last step with a faster release speed until liquid starts dropping from the loading port
9. Wait 5 minutes (while preparing library for loading)
    - 15 µl Sequencing Buffer (SB)
    - 10 µl Library Beads, mixed ***by pipetting*** immediately before use
    - 5 µl DNA library
    - mix ***by pipetting***
10. Remove the tape from the waste port
11. Drop another 30 µl of flush solution onto the loading port, wait for it to drain through
12. Drop 30 µl of sequencing library onto the loading port, wait for it to drain through
13. Re-seal the flow cell by rolling a finger across the plastic adhesive cover, trying to avoid putting pressure on the flow cell matrix

Loading – MinION Flow Cells

This method uses BSA (can probably be omitted), with a library load amount of 12 µl in 75 µl:

1. Prepare Flow Cell Flush solution:
    - 1170 µl Flow Cell Flush (FCF), i.e. an entire tube
    - 12.5 µl Bovine Serum Albumin (BSA) at 20 mg/µl [recombinant BSA seems to work as well]
    - 30 µl Flow Cell Tether (FCT)
    - mix ***by pipetting***
2. Load 800µl flush solution into ***Priming port***
3. Wait 5 minutes (while preparing library for loading)
    - 37.5 µl Sequencing Buffer (SB)
    - 25.5 µl Library Beads (LIB), mixed ***by pipetting*** immediately before use
    - 12 µl DNA library
    - mix ***by pipetting***
4. Lift up SpotON port cover
5. Load 200µl flush solution into ***Priming port***
6. Add 75µl library dropwise into ***SpotON port***

Loading – PromethION Flow Cells

BSA is not used for PromethION flow cell loading, and the library load amount is 12 µl in 200µl:

1. Prepare Flow Cell Flush solution:
    - 1170 µl Flow Cell Flush (FCF), i.e. the entire tube
    - 30 µl Flow Cell Tether (FCT)
    - mix ***by pipetting***
2. Mix by pipetting
3. Load 500µl flush solution into inlet port
4. Wait 5 minutes (while preparing library for loading)
    - 100 µl Sequencing Buffer (SB)
    - 68 µl Library Beads (LIB), mixed ***by pipetting*** immediately before use
    - 12 µl DNA library
    - 20 µl Elution Buffer (EB)
    - mix ***by pipetting***
5. Lift up SpotON port cover
6. Load 500µl flush solution into inlet port
7. Load 200µl library into inlet port by slow pipetting