Protocol C-circle assay - AG Rippe

1. DNA concentration
   - Measure DNA concentration with Qubit dsDNA HS Assay (Life Technologies)
   - Dilute samples to a concentration of 10-30 ng/µl

2. Amplification
   - Optional: Digest DNA with HinfI/RsaI and remove RNA by RNase digest
   - Prepare 30 ng of each sample in 10 µl H2O (every sample twice to include no Pol controls for sample)
   - Prepare Master mix (without addition of polymerase first, see below)

	Stock	Final conc.	Vol per sample
BSA (NEB)	10 mg/ml	0.2 mg/ml	0.2 µl
Tween 20	10%	0.1 %	0.1 µl
dATP, dGTP, dTTP (Invitrogen)	100 mM	1 mM each	0.1 µl each
Φ29 buffer (NEB)	10x	2x	2 µl
Nuclease-free water		Ad 10 µl	6.65 µl
Φ 29 polymerase (NEB)	10 U/µl	7.5 U	0.75 µl

   - **Before addition of polymerase: add 9.25 µl Master mix to no Pol controls!**
   - Add 10 µl Master mix with polymerase to all other samples and mix
   - In PCR thermocycler:
     - 8 h @ 30°C
     - 20 min @ 65°C
     - 4°C hold

3. Dot Blot with Dot blotter (Bio Rad)
   - Add 60 µl 2X SSC to amplified samples in PCR tubes (total volume 80 µl)
   - Cut Nylon membrane (Roti-Nylon plus, 0.45 µm) and soak 5 min in 2X SSC
   - Place membrane on dot blot apparatus without trapping air bubbles!
   - Load ~100 µl 2X SSC in all wells and apply vacuum
   - Load samples and fill empty wells with 2X SSC
   - Apply vacuum
   - Reload all wells with 100 µl 2X SSC and apply vacuum again

- Let membrane dry for 10 min
- Bake membrane on whatman paper in glass dish for 20 min at 120 °C
- Wash membrane once in 2X SSC
- At this step the membrane can be dried and stored at 4 °C for several days
- Hybridization with DIG-telomere probe according to Telo TAGGG telomere length kit (Roche); in short (for each washing step prepare ~30 ml):
    - Prewarm DIG Easy Hyb Granules (bottle 7) to 42 °C
    - Prehybridization: Cover the membrane with prewarmed DIG Easy Hyb Granules in a tupper box and incubate for 30-60 min at 42 °C with gentle agitation
    - Prepare 5-10 ml hybridization solution depending on size of membrane (1 µl telomere probe per 5 ml prewarmed DIG Easy Hyb Granules)
    - Discard prehybridization solution and immediately add hybridization solution to the membrane
    - Incubate for 3 h (or O/N) at 42 °C with gentle agitation
    - Discard hybridization solution
    - Wash membrane twice for 5 min at RT with stringent wash buffer I (2X SSC, 0.1% SDS)
    - Wash membrane twice for 15-20 min at 50 °C with prewarmed stringent wash buffer II (0.2X SSC, 0.1% SDS) with gentle agitation
    - Rinse membrane in at least 30 ml 1X washing buffer for 5 min at RT with gentle agitation
    - Prepare ~60 ml 1X blocking solution: Dilute maleic acid buffer (bottle 12) 1:10 with H_2O to get a 1X solution. Then, dilute 10X blocking buffer (bottle 11) 1:10 with1X maleic acid buffer.
    - Incubate membrane in 30 ml freshly prepared 1X blocking solution for 30 min at RT with gentle agitation
    - Prepare Anti-DIG-AP working solution: Spin vial for 5 min at 13,000 rpm before use. Dilute Anti-DIG-AP (bottle 13) with 1X blocking solution to a final concentration of 75 mU/ml (1:10,000)
    - Incubate the membrane in 30 ml Anti-DIG-AP working solution for 30 min at RT with gentle agitation
    - Wash membrane 2x 15 min at RT with 1X washing buffer (bottle 10; 50 ml each time) with gentle agitation

- Prepare 1X detection buffer: Dilute 10X detection buffer (bottle 14) 1:10 with H_2O
- Incubate membrane in 30 ml of 1X detection buffer for 2-5 min at RT with gentle agitation
- Discard detection buffer and remove excess liquid by placing the membrane on a whatman paper. Don’t let the membrane dry!
- Place the wet membrane, DNA side facing up, on an open transparent sheet and very quickly apply about 2 ml substrate solution (bottle 15) to the membrane
- Immediately cover the membrane with the transparent sheet and spread the substrate solution without trapping air bubbles
- Incubate the membrane for 30 min to several hours at RT
- Squeeze out excess substrate solution
- Expose the membrane in a Bio-Rad Chemi-Doc MP System (Program “Chemi High Resolution”)