

Protocol Supplement to Castle *et al*, Genome Biology 2003, 4:R66

Reaction	Amount
Perform RP-RT#1	
Thaw 1.33 μ m shDNP256 primer plate on ice	
Resuspend_shDNP256	15 μ L
Incubate at room temperature	15 minutes
RunPCR rp-rt-a	10min70c>5min4c>10min22c
Complete RT #1 Cocktail, add, and mix	1.5X
RT#1 Premix	30 μL
0.1 M DTT	6 μL
Superscript II	1.5 μL
Add_RT1_Cocktail	25 μ L
Quick Spin	20 sec. x 500 rcf
Run PCR rp-rt-60min	60min42c>4c hold
Hydrolyze RNA	
RPamp_RT1_Add_NaOH_EDTA	20 μ L
Run PCR rp-rt-c	20min65c>4c hold
RPamp_RT1_Add_Trис	20 μ L
Add_H2O_RT1	20 μ L
Purify the cDNA	Qiaquick
Dry	
Dry down remaining volume in Speed-Vac	2 hours x 50C
Rehydrate & Perform 2ss	
Thaw 1.35 μ m shT7N9 primer plate on ice	
Resuspend_shT7N9 primer	20 μ L
Incubate at Room Temperature	15 minutes
RunPCR second-str-1	5min70c>10min22c>4c hold
Complete 2ss Cocktail, add, and mix	1.5X
Premix	43.5 μL
5 U/ μ L Klenow (Large Fragment Polymerase)	1.5 μL
Add_SS_Cocktail	30 μ L
Run PCR second-str-2	1hr37c>2min65c>4c hold
Purify the DNA	Qiaquick
Dry	
Dry down remaining volume in Speed-Vac	2 hours x 50C
Rehydrate & Perform PCR	
Resuspend_PCR	25 μ L water
Incubate at Room Temperature	15 minutes
Perform PCR Reaction	
Thaw PCR Cocktail on ice	
Complete PCR cocktail, add, and mix	1.5X
Premix	106.5 μL
T7 Primer (100uM)	1.5 μL
DP256 Primer (100uM)	1.5 μL
Taq Polymerase	3 μL
Add_PCR_Cocktail	75 μ L
Run PCR program	
Purify the dsDNA	Qiaquick
Dry	
Dry down remaining volume in Speed-Vac	2 hours x 50C
Rehydrate & Perform IVT	
Resuspend_IVT	40 μ L water
Incubate at Room Temperature	15 minutes
Complete IVT cocktail, add, and mix	1.5X
Premix	48.15 μL
100 mM DTT	9 μL
RNAGuard	0.75 μL
T7 Polymerase	1.2 μL
iPPase	0.9 μL
Add_IVT_Cocktail	40 μ L

Run PCR DTAP-IVT	16hrs42c>4c hold
Purify the cRNA	RNeasy
Quantify cRNA	
Generate RP-RT#2 Plates	
Split cRNA	3ug/well
Dry	
Dry down remaining volume in Speed-Vac	20 Minutes x 50C
Resuspend and anneal to Primer	
Thaw N9 Primer Source Plate	
RP-Amp_Resuspend_N9	15 µL
Incubate at room temperature	15 minutes
RunPCR rp-rt-a	10min70c>5min4c>10min22c
RT Reaction	
Complete RT Cocktail and add mix:	1.5X
Premix	41.25 µL
100 mM DTT	7.5 µL
200 U/µL SSII	3.75 µL
Distribute 96-well plate	80 µL
RP-Amp_Add_RT2Cocktail	35 µL
Run PCR rp-rt-60min	60min42c>4c hold
Hydrolysis and Neutralization	
RP-Amp_RT2_Add_NaOH_EDTA	25 µL
Run PCR rp-rt-c	20min65c>4c hold
RP-Amp_RT2_Add_Trис	25 µL
RP-Amp_Add_NaOAc	7 µL
Purify the ssDNA	Qiaquick
Quantify	
Pool/Concentrate Samples	
Quantify concentrated aa-ccDNA	
Create Coupling Ready Plates	
Split samples into coupling ready plates	5 µg
Dry down volume in Speed-Vac	30 minutes x 50C

Premixes contain buffers and nucleotides

Cocktails are premixes with enzymes and DTT

Premixes made be made ahead of time and frozen at -20C. Make the cocktail by thawing and adding the enzymes and DTT just before use.

RT1 Final Reaction Concentrations

10 mM DTT, 50 mM Tris-HCl pH 8.3, 75 mM KCl, 8 mM MgCl₂, 0.5 mM dNTPs, 5 U/μl Superscript II

Second Strand Final Reaction Concentrations

0.2 mM DTT, 2.1 mM Tris-HCl pH 7.9, 2.1 mM MgCl₂, 10.7 mM NaCl, 1.07 mM dNTPs, 0.1U/μl Klenow

PCR Final Reaction Concentrations

20 mM Tris-HCl pH 8.4, 50 mM KCl, 0.01 mM dNTPs, 1.5 mM MgCl₂, 0.01 U/μl Taq Polymerase

IVT Final Reaction Concentrations

7.5 mM DTT, 40 mM Tris-HCl pH 7.5, 14.25 mM MgCl₂, 10 mM NaCl, 2 mM Spermidine, 125 U/ml RNAGuard, 2.5 mM dNTPs, 15 U/ml IPPase, 25kU/ml T7 Polymerase

RT2 Final Reaction Concentrations

10 mM DTT, 50mM Tris-HCl pH 8.3, 75 mM KCl, 8 mM MgCl₂, 0.5 mM dNTPs, 0.5 mM aa-dUTP, 5 U/μl Superscript II

PCR Conditions:

1. 94C for 5 min
 2. 94C for 45 sec
 3. 40C for 2 min
 4. 72C for 4 min
- Repeat 2-4 one time
5. 94C for 45 sec
 6. 55C for 2 min
 7. 72C for 4 min
- Repeat 5-7 seven times
- 4C forever