prepIT®•L2P

Laboratory protocol for manual purification of DNA from whole sample

Ethanol precipitation protocol and prepIT®•L2P reagent for the purification of genomic DNA from Oragene® products and ORAcollect® formats OC-175, OCD-100 and OCR-100. Not for use with OCD-100A.

Visit our website at www.dnagenotek.com for any additional languages and protocols.

Note: This protocol requires the use of a centrifuge capable of generating at least 3,500 × *g* to obtain optimal results.

The procedure is described for purifying DNA from the entire collected sample (approximately 1 mL to 4 mL total volume). The volumes shown should be adjusted for the actual collected volume.

Reagents included

- prepIT•L2P (catalog #: PT-L2P)

Equipment and reagents

- Centrifuge that accommodates 15 mL tubes, and is capable of generating at least 3,500 × *g* (see Table 2)
- 15 mL conical polypropylene tubes (e.g., BD Falcon #352196)
- Microcentrifuge capable of running at 15,000 × *g* (optional)
- 1.5 mL microtubes (e.g., Axygen #MCT-150-C)
- Air or water incubator at 50°C
- Ethanol (95% to 100%) at room temperature
- Ethanol (70%) at room temperature
- DNA storage buffer: TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) or similar solution

Pre-purification check

Weigh the sample to estimate the amount of saliva provided by the donor (see Table 1; not required for ORAcollect products). The amount of saliva collected is directly proportional to the amount of DNA recovered. As an example, if a donor has provided less than 2 mL of saliva, you should expect to recover a lower total yield from this sample. Correspondingly, a donor providing more than 2 mL of saliva should result in higher total yield.

Weight of kit (without sample)

Once a sample arrives at the lab, we suggest weighing the sample to estimate if the right amount of saliva was provided by the donor. You can expect some variability across donors as determined by weight of triggered kit with sample. The average weight of an empty kit is provided (Table 1). To calculate the amount of sample collected (assuming 1 g/mL), perform the following subtraction:

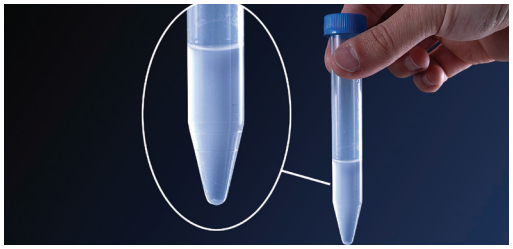
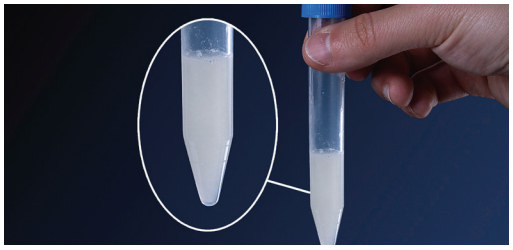
Weight of kit containing sample - Weight of kit without sample = Amount of sample collected

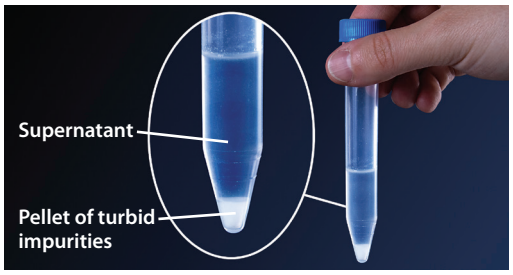
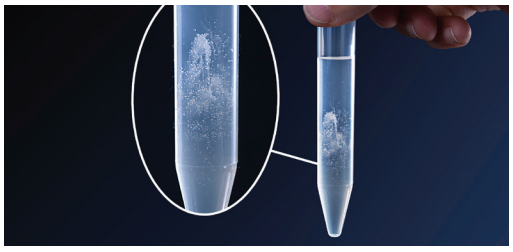
Table 1

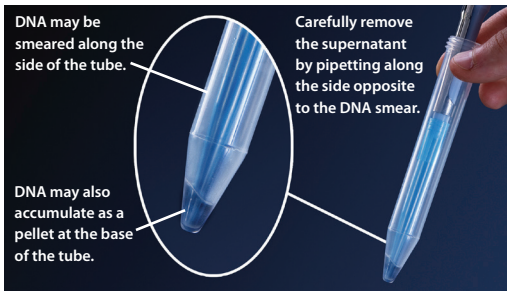
Product #	Weight of kit without sample
OG-250/OGR-250	14.15 g
OG-500/OGD-500/OGR-500/OG-600*/ OGD-600*/OGR-600*	6.81 g
OG-510/OGD-510/OG-610*/OGD-610*	5.83 g
OG-520	5.41 g
OG-575/OGD-575/OGR-575/OG-675*/ OGD-675*/OGR-675*	5.66 g
ON-500/ON-600*	6.47 g

* 2D bottom barcoded kits are 1 g heavier than the respective non-barcoded kit.

Procedure

Purification steps	Notes
1. Mix the sample in the DNA Genotek kit by inversion and gentle shaking for a few seconds.	• This is to ensure that viscous samples are properly mixed.
2. Incubate the sample at 50°C in a water incubator for a minimum of 1 hour or in an air incubator for a minimum of 2 hours. **Note:** The use of an air incubator may be preferable since the sample tubes may float in a water bath. If a water bath must be used, ensure the sample-containing portion of the tube remains immersed in water.	• This heat-treatment step is essential to maximize DNA yield and ensure that nucleases are permanently inactivated. • This must be done in the original collection tube. • The sample may be incubated at 50°C overnight if more convenient. • This incubation step may be performed at any time after sample is collected and before DNA is purified. • A longer time is required in an air incubator because temperature equilibration is slower than in a water incubator.
3. Transfer the entire sample to a 15 mL centrifuge tube (Figure 1). Note the volume of the sample.  ***Figure 1**: Before proceeding to Step 4, ensure that the entire sample has been incubated and transferred to a fresh 15 mL centrifuge tube, as shown.*	• Transfer can be carried out either by pouring or by pipetting with a glass or plastic pipette.
4. Add 1/25th volume of PT-L2P and mix by vortexing for a few seconds. (Figure 2)  ***Figure 2**: After adding the PT-L2P and incubating on ice for 10 minutes, the sample will no longer look clear, but rather a cloudy solution.*	• e.g., 160 µL for 4 mL of sample to each tube. • The sample will become turbid as impurities and inhibitors are precipitated.
5. Incubate on ice for 10 minutes.	• Room temperature incubation can be substituted but will be less effective at removing impurities.

Purification steps	Notes
6. Centrifuge at room temperature for 10 minutes at as high a speed as is possible. Minimum 3,500 x *g*. Any centrifuge, either swing-out bucket or angle rotor that can generate this *g*-force is suitable.  ***Figure 3**: After centrifugation, there will be an accumulation of turbid material at the base of the tube. The supernatant should be visibly clear.*	• Higher centrifugal force minimizes the amount of turbid material that will be carried over into the purified DNA (Figure 3). • Before proceeding, you should verify with the tube manufacturer that the 15 mL centrifuge tubes can withstand the centrifugal force. • A longer period of centrifugation (up to 20 minutes) can be carried out where reducing the turbidity of the final DNA solution is considered to be important.
7. Carefully transfer the majority of the clear supernatant with a pipette to a fresh 15 mL centrifuge tube. **Discard the pellet.**	• Leave a small volume of the supernatant behind to avoid disturbing the pellet. The pellet contains turbid impurities.
8. Add 1.2x volume of room temperature 95% to 100% ethanol to the clear supernatant. Mix gently by inversion 10 times.  ***Figure 4**: After the addition of ethanol, the DNA will precipitate which **may** result in a visible clot of fibres.*	• During mixing with ethanol, the DNA will be precipitated. Precipitated DNA may appear as a clot of DNA fibres (Figure 4). Even if no clot is seen, DNA will be recovered in the following steps.
9. Let the sample stand at room temperature for 10 minutes to allow the DNA to fully precipitate.	• Incubation at -20°C is not recommended because impurities may co-precipitate with the DNA.
10. Centrifuge at room temperature for 10 minutes at as high a speed as is possible. Minimum 3,500 × *g*.	• A minimum centrifuge speed of 3,500 × *g* (see Table 2) is required. Any centrifuge, either swinging-bucket or angle rotor that can generate this *g*-force is suitable.

Purification steps	Notes
11. Carefully remove the supernatant with a glass or plastic pipette and discard it. Take care to avoid disturbing the DNA pellet.  ***Figure 5**: Using a pipette tip to gently scratch along the inside of the tube may reveal the presence of a DNA smear.*	• The supernatant may contain impurities and should be removed as completely as possible. • Precipitated DNA will be found as a pellet at the bottom of the tube and possibly as a smear down the side of the tube (Figures 5). • The DNA smear may be located on the side of the tube facing away from the centre of the centrifuge. • A smear can be located using the "scratch" test. You can check for the presence of a DNA smear by scratching the inside of the tube using a P1000 tip. A smear, as shown in figure 5, may be visible.
12. Ethanol wash: Carefully add 1 mL of 70% ethanol to the tube without disturbing the smear or the pellet. Let it stand at room temperature for 1 minute. Gently swirl and **completely remove the ethanol, being careful not to disturb the pellet and the smear**.	• **It is important to remove all ethanol from the sample. Carryover of ethanol may impact the performance of the assay.** • Take care not to disturb the DNA pellet or the smear. • A short centrifugation (less than 1 minute) can be performed to facilitate complete removal of the supernatant. • Should the pellet detach after the ethanol wash step, centrifuge the sample for 5 minutes at as high a speed as is possible. Minimum 3,500 x *g*.
13. Rehydrate the DNA by adding 0.2 – 1 mL of TE solution and by vortexing the sample for 30 seconds. For ORAcollect products, rehydrate the DNA by adding 0.2 mL of TE solution and vortex the sample for 30 seconds.  ***Figure 6**: Vortexing the sample for 30 seconds will allow you to recover DNA smeared on the side of the tube. The DNA will remain high molecular weight.*	• If a higher concentration of DNA is desired, the volume of TE may be reduced. A minimum of 200 µL TE solution should be used. • Excessive drying of the pellet (> 10 minutes) and using less than 500 µL of TE solution can make it difficult to rehydrate (dissolve) the DNA and may decrease the yield or make quantification difficult. • Precipitated DNA will be found as a pellet at the bottom of the tube and possibly as a smear down the side of the tube. • To ensure maximum DNA recovery, the sample must be vortexed after the addition of DNA solvent (TE solution). Vortexing will ensure that the DNA smeared on the side of the tube is recovered (Figure 6). • Do not hesitate to vortex the sample as the DNA will remain high molecular weight.

Purification steps	Notes
14. To ensure complete rehydration of the DNA (pellet and smear) incubate at room temperature overnight followed by vortexing or at 50°C for 1 hour with occasional vortexing.	• Incomplete rehydration of the DNA is a cause of inaccuracy in estimating DNA concentration and potential failure of downstream applications such as PCR.
15. Transfer the rehydrated DNA to a 1.5 mL microcentrifuge tube for storage.	
Optional step: a) Centrifuge the rehydrated DNA at room temperature for 15 minutes at 15,000 x *g*. b) Transfer the supernatant to a fresh 1.5 mL microcentrifuge tube without disturbing the pellet.	Note that the pellet contains insoluble, turbid material. • To maximize DNA recovery, ensure that the DNA is completely rehydrated (step 14) prior to performing this centrifugation step. • This centrifugation step ensures that any remaining turbid material is removed from the DNA sample. • Care should be taken not to disturb the pellet when transferring the clear supernatant to a fresh tube.
16. Options for storage of the fully rehydrated DNA: a) **Recommended** in TE, in aliquots at -20°C for long-term storage, or b) In TE at 4°C for up to 2 months.	• Freezing of purified DNA in TE may cause the DNA to precipitate. When thawing frozen purified DNA, pay careful attention to rehydration, as discussed in step 14.

Quantification of DNA

By fluorescence method

Assays that use fluorescent dyes are more specific than absorbance at 260 nm for quantifying the amount of double-stranded DNA (dsDNA) in a DNA sample. We recommend using fluorescent dyes such as PicoGreen® or SYBR® Green I to quantify dsDNA since there is less interference by contaminating RNA. An inexpensive protocol using SYBR Green I is described in PD-PR-075, *DNA quantification using SYBR Green I Dye and a micro-plate reader*[1]. Alternatively, commercially available kits such as Invitrogen's Quant-iT™ PicoGreen dsDNA Assay Kit (Cat. No. Q-33130) can be used. For either protocol, we recommend that the purified DNA be diluted 1:50 with TE solution and that 5 µL be used in the quantification assay.

By absorbance method

If you choose to quantify DNA by absorbance, we recommend that you first treat the purified sample with RNase to digest contaminating RNA and then remove the RNA fragments by ethanol precipitation of the DNA. A detailed protocol is described in PD-PR-040, *RNA removal by double-RNase digestion*[2]. Please note that DNA from oral sample typically contains appreciably more RNA than found in blood samples. Ensure that alcohol-precipitated DNA is fully dissolved before reading the absorbance.

Conversion factor: An absorbance of 1.0 at 260 nm corresponds to a concentration of 50 ng/µL (50 µg/mL) for pure dsDNA.

Ensure that absorbance values are within the linear range of the spectrophotometer. Re-dilute and re-measure samples that fall outside of the linear range. See your instrument documentation for more information.

Method:

1. Dilute a 10 µL aliquot of purified RNase treated DNA with 90 µL of TE (1/10 dilution). Mix by gently pipetting up and down. Wait for bubbles to clear.
2. Use TE in the reference (blank) cell.
3. Measure absorbance at 320 nm, 280 nm and 260 nm.
4. Calculate corrected A_{280} and A_{260} values by subtracting the absorbance at 320 nm (A_{320}) from the A_{280} and A_{260} values.
5. DNA concentration in ng/µL = corrected $A_{260} \times 10$ (dilution factor) $\times 50$ (conversion factor).
6. A_{260}/A_{280} ratio: Divide corrected A_{260} by corrected A_{280}.

Example

1. Assume the measured A_{320}= 0.025, A_{280}= 0.175 and A_{260}= 0.295
2. The DNA concentration of the undiluted sample will be:
   $(A_{260} - A_{320}) \times 10$ [dilution factor] $\times 50$ [conversion factor]
   $= (0.295 - 0.025) \times 10 \times 50$
   $= 0.270 \times 10 \times 50$
   = 135 ng/µL or 135 µg/mL
3. The corrected A_{260}/A_{280} ratio will be:
   $(A_{260} - A_{320}) \div (A_{280} - A_{320})$
   $= (0.296 - 0.025) \div (0.175 - 0.025)$
   $= 0.270 \div 0.150$
   = 1.80

Table 2: Calculation of *g*-force from radius and speed of rotor

In order to generate a minimum *g*-force of 3,500 x *g*, you must select a spin speed (RPM) that is appropriate for the size of your rotor. Highlighted below are the combinations of spin speed and rotor radius that will generate the minimum g-force required.

e.g., If your centrifuge has a rotor radius of 10 cm you must select a **minimum** spin speed of 6,000 RPM. Remember that 3,500 x *g* is the minimum requirement. You should spin your samples at as high a speed as your centrifuge can support, and producing a force that your tubes can withstand.

Radius of rotor (cm)												
RPM	7	8	9	10	11	12	13	14	15	16	17	18
4,000	1,253	1,432	1,611	1,790	1,049	1,969	2,148	2,328	2,507	2,865	3,044	3,223
4,500	1,586	1,813	2,039	2,266	2,493	2,719	2,946	3,172	3,399	3,626	3,852	4,079
5,000	1,958	2,238	2,518	2,798	3,077	3,357	3,637	3,917	4,196	4,476	4,756	5,036
5,500	2,369	2,708	3,046	3,385	3,723	4,062	4,400	4,739	5,077	5,416	5,754	6,036
6,000	2,820	3,223	3,626	4,028	4,431	4,834	5,237	5,640	6,043	6,445	6,848	7,251
6,500	3,309	3,782	4,255	4,728	5,201	5,673	6,146	6,619	7,092	7,564	8,037	8,510
7,000	3,838	4,386	4,935	5,483	6,031	6,580	7,128	7,676	8,225	8,773	9,321	9,870
7,500	4,406	5,036	5,665	6,294	6,924	7,553	8,183	8,812	9,442	10,071	10,700	11,330
8,000	5,013	5,729	6,445	7,162	7,878	8,594	9,310	10,026	10,742	11,459	12,175	12,891
8,500	5,659	6,468	7,276	8,085	8,893	9,702	10,510	11,319	12,127	12,936	13,744	14,553
9,000	6,345	7,251	8,158	9,064	9,970	10,877	11,783	12,689	13,596	14,502	15,409	16,315
***g*-force**												

References

1 DNA quantification using the Fluorescence/DNase (F/D) assay. Replaced by DNA quantification using SYBR Green I dye and a micro-plate reader. DNA Genotek. PD-PR-075.

2 RNA removal by double-RNase digestion. DNA Genotek. PD-PR-040.

Technical support is available Monday to Friday (9h00 to 17h00 EST):

- Toll-free (North America): 1.866.813.6354, option 6
- All other countries: 613.723.5757, option 6
- Email: support@dnagenotek.com

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prepIT®•L2P

Quick reference guide:
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	1 mL	1.5 mL	2.0 mL	2.5 mL	3 mL	3.5 mL	4 mL
prepIT•L2P	40 µL	60 µL	80 µL	100 µL	120 µL	140 µL	160 µL
95 % to 100% EtoH	1.2 mL	1.8 mL	2.4 mL	3 mL	3.6 mL	4.2 mL	4.8 mL
70 % EtoH	← 1 mL →						
TE	← 0.2 mL to 1 mL →						

Purification steps
1. Mix the sample in the DNA Genotek kit by inversion and gentle shaking for a few seconds.
2. Incubate the sample at 50°C in a water incubator for a minimum of 1 hour or in an air incubator for a minimum of 2 hours.
3. Transfer the entire sample to a 15 mL centrifuge tube. Note the volume of the sample.
4. Add 1/25th volume of PT-L2P and mix by vortexing for a few seconds.
5. Incubate on ice for 10 minutes.
6. Centrifuge at room temperature (RT) for 10 minutes at as high a speed as is possible. Minimum 3,500 x *g*.
7. Carefully transfer the majority of the clear supernatant with a pipette to a fresh 15 mL centrifuge tube. **Discard the pellet.**
8. Add 1.2x volume of RT 95% to 100% ethanol to the clear supernatant. Mix gently by inversion 10 times.
9. Let the sample stand at RT for 10 minutes to allow the DNA to fully precipitate.
10. Centrifuge at RT for 10 minutes at as high a speed as is possible. Minimum 3,500 × *g*.
11. Carefully pipette off the supernatant and discard it. Take care to avoid disturbing the DNA pellet.
12. Add 1 mL of 70% ethanol to the tube without disturbing the pellet or smear. Let it stand at RT for 1 minute. Gently swirl and **completely remove the ethanol, without disturbing the pellet or smear.**
13. Rehydrate the DNA by adding 0.2 – 1 mL of TE solution and vortexing the sample for 30 seconds. For OC-100 and OCR-100, rehydrate the DNA with 0.2 mL of TE.
14. Incubate overnight at RT or at 50°C for 1 hour.
15. Vortex sample and transfer the rehydrated DNA to a fresh 1.5 mL microcentrifuge tube for storage.
16. Optional: Centrifuge DNA at RT for 15 minutes at 15,000 x g then transfer the supernatant to a fresh 1.5 mL microcentrifuge tube without disturbing the pellet.
17. Storage: In aliquots at -20°C for long-term storage (recommended) or at 4°C for up to 2 months.