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Introduction

E.Z.N.A.[®] Blood DNA Midiprep Kits are designed for isolation of genomic DNA from up to 10 ml of fresh, whole blood treated with any common anticoagulant such as heparin, EDTA, or acid-citrate-dextrose. 10 ml of blood typically yields 200–250 µg of total DNA. The procedure completely removes contaminants and enzyme inhibitors making total DNA isolation fast, convenient, and reliable. There is no need for phenol/chloroform extractions, and time-consuming steps such as CsCl gradient ultracentrifugation, and precipitation with isopropanol or LiCl, are eliminated. DNA purified using the E.Z.N.A.[®] Blood DNA method is ready for applications such as RT-PCR*.

Principle

The E.Z.N.A.[®] Blood DNA Kits use the reversible binding properties of HiBind[®] matrix, a new silica-based material. This is combined with the speed of midi-column spin technology. Red blood cells are selectively lysed and white cells collected by centrifugation. After lysis of white blood cells under denaturing conditions that inactivate DNases, genomic DNA is purified on the HiBind[®] Midi spin column. A specifically formulated high salt buffer system allows DNA molecules greater than 200 bases to bind to the matrix. Cellular debris and other contaminants (such as hemoglobin) are effectively washed away and high quality DNA is finally eluted in DNA Elution Buffer.

Storage

E.Z.N.A.[®] Blood DNA Midiprep Kits should be stored at room temperature. During shipment crystals may form in the BL Lysis Buffer. Warm to 37°C to dissolve. All the components are guaranteed for at least 24 months from date of purchase.

*The PCR process is covered by U.S. Patents 4,683,195 and 4,683,202 (and international equivalents) owned by Hoffmann-LaRoche, Inc.

Kit Contents

| E.Z.N.A. [®] Blood DNA Kits | DNA Preps 2 | DNA Prep 10 | DNA Prep 25 |
|--------------------------------------|----------------------|----------------------|----------------------|
| Product Number | D3493-00 D3494-00 | D3493-01 D3494-01 | D3493-02 D3494-02 |
| Purification | 2 | 10 | 25 |
| HiBind™ DNA Midi Columns | 2 | 10 | 25 |
| 8 ml Collection Tubes | 4 | 20 | 50 |
| Buffer ERL, 10 X Concentrate | 20ml | 100 ml | 2 x 200 ml |
| Buffer TL | 5ml | 25 ml | 60 ml |
| Buffer BL | 5ml | 25 ml | 60 ml |
| Buffer HB | 7 ml | 40 ml | 80 ml |
| DNA Wash buffer Concentrate | 12ml | 2 x 40 ml | 2 x 100 ml |
| OB Protease (D3494 only) | 6mg | 30 mg | 75 mg |
| RNase A | 45µl | 210µl | 520µl |
| DNA Elution Buffer | 10ml | 40 ml | 100 ml |
| Instruction Manual | 1 | 1 | 1 |

Harvesting and Storage of Blood

E.Z.N.A.[®] DNA Midiprep Kits are designed for purification of genomic DNA from up to 10 ml whole blood. The system is not limited by DNA binding capacity of HiBind[®] Midi columns (which can bind up to 500 ug of DNA), but by lysis volume. Due to the abundance of erythrocytes and proteins, greater than 20 ml whole blood will significantly lower DNA quality. The relatively low DNA content of leukocytes means that the maximum binding capacity of HiBind[®] DNA columns can not be reached.

Samples should be collected in the presence of an anticoagulant (preferably acid-citrate-dextrose) and processed within a few hours. Minimize storage prior DNA isolation as leukocyte transcripts generally have variable stabilities.

Before Starting

| | | |
|------------------|-----------------------|--|
| IMPORTANT | 1. | Buffer ERL is supplied as a 10 X concentrate and must be diluted with sterile deionized water as follows. |
| | D3493-00 & D3494-00 | Dilute with 180ml deionized water per bottle |
| | D3493-01 and D3494-01 | Empty contents of the bottle supplied into an appropriately sized vessel and add 900 ml deionized water per bottle of Buffer ERL. |
| | D3493-02 & D3494-02 | Empty contents of the bottle supplied into an appropriately sized vessel and add 1800 ml deionized water per bottle of Buffer ERL. |
| | 2. | D3494: Reconstitute OB Protease in 0.3ml (2 preps), 1.5 ml (10 Prep) and 3.75 ml for (25 preps) 10 mM Tris-HCl, pH 8. Vortex vial briefly prior to use. D3493: Prepare a 20 mg/ml solution of molecular biology grade Proteinase K in 10 mM Tris-HCl, pH 7.4. We recommend that you aliquot and store vials of reconstituted protease at -20°C. |
| | 3. | DNA Wash buffer must be diluted with absolute ethanol before use. |
| | D3493-01&D3494-01 and | Add 60 ml 100% ethanol to each bottle |
| | D3493-02 & D3494-02 | Add 150 ml 100% ethanol to each bottle |

All centrifugation steps must be carried out at room temperature.

Please take a few minutes to read this booklet thoroughly and become familiar with the protocol. Prepare all materials required before starting to reach optimize performance.

- All the subsequent steps must be performed using a centrifuge capable of at least 8000

x g. Ensure that an appropriate rotor adaptor is in place to prevent damage to the collecting tube.

- **Water bath** - set to 70°C.
- **Absolute ethanol** - approximately 3 ml per sample
- **Proteinase K** - For D3493 prepare a stock solution of Proteinase K at 20 mg/ml; for D3494, prepare a stock solution of OB pretease at 20mg/ml.
- Have a shaking water bath set to 55°C.
- 50 ml centrifuge tubes and clean 15-20 ml centrifuge tubes.
- Laboratory centrifuge equipped with **swinging-bucket** rotor.

Standard Protocol (for 2 ml of whole blood sample)

This protocol allows genomic isolation from up to 2 ml blood sample. Yield vary depend on source.

Add up to 2 ml whole blood to a 15 ml centrifuge tube. If the sample less than 2 ml, bring the volume up to 2 ml with 10 mM Tris-HCl, PBS, or Elution Buffer provided.

1. For 2 ml whole blood sample, add 150 µl OB Protease (D3494) or proteinase K (20mg/ml) and mix the sample by vortexing. Add 2.1 ml of Buffer BL. Vortex 5 minutes to mix thoroughly.
2. Add 20 µl RNase A solution to each sample to remove RNA.
3. Incubate sample at 70°C for 10 min.
4. Briefly vortex the tube once during incubation.
5. Add 2.2 ml of isopropanol to lysate and mix. **For buffy coat, isolated leukocytes, and cultured cells, yields will improve if 260 µl absolute ethanol is used in place of isopropanol.**
6. Insert a HiBind[®] DNA Midi-spin column in a 15 ml collection tube (provided). Transfer the solution from Step 6 into the column and centrifuge at 4,000 x g for 5 min to bind DNA. **Discard the collection tube and flow-through liquid.**
7. Place the column into a **second 15 ml tube** and wash by pipetting 3 ml of HB Buffer. Centrifuge at 4,000 x g for 5 minutes Discard the flow-through liquid and re-use the collection tube.
8. Place the column into a **same 15 ml tube** from step 8 and wash by pipetting 4 ml of DNA Wash Buffer diluted with ethanol. Centrifuge at 4,000 x g for 5 minutes. Discard the flow-through liquid and re-use the collection tube.
9. Using a **same centrifuge tube from step 8**, wash the column with a second 4 ml of Wash Buffer and centrifuge as above. Discard flow-through.
10. Using the same 15 ml collection tube, centrifuge at 4000 x g for 10 min to dry the column. **This step is critical for removal of trace ethanol that might otherwise interfere with downstream applications.**
11. Place the column into a nuclease-free 15 ml centrifuge tube (not provided) and add 0.5 ml of preheated (70°C) Elution Buffer. Allow tubes to sit for 2 min at room temperature.

12. To elute DNA from the column, centrifuge at 4,000 x g for 5 min. Retain flow-through containing the DNA. Place column into a second 1.5 ml tube and repeat elution step with another 500 µl of preheated Elution Buffer. Discard column.
Note: Each elution typically yields 60%-70% of the DNA bound to the column. Thus two elutions generally give >80%. However, increasing elution volume reduces the concentration of the final product. To obtain DNA at higher concentrations, elution can be carried out using same 0.5 ml Elution to perform second elution.

Maximum Yield Protocol (for up to 10 ml whole blood)

1. To 1 volume of whole fresh blood (maximum of 10 ml) add 5 volumes of 1 x Buffer ERL. For example add 5 ml Buffer ERL to 1 ml blood. Mix by vortexing.

Note: Buffer ERL is supplied as a 10 x concentrate and must be diluted with sterile deionized water before use. Refer to page 4 or label on bottle for directions.

2. Incubate for 15 min on ice, mixing by brief vortexing twice. Lysis of red blood cells is indicated when the solution becomes translucent. Blood samples from individuals with an elevated hematocrit or ECR, extend incubation time to 20 min.
 3. Pellet leukocytes by centrifuging at 450 x g for 10 min at 4°C. Completely remove and discard the supernatant containing lysed red blood cells.

4. Wash the white blood cell pellet with 2 volumes of Buffer ERL per volume of whole blood used in step 1. Thoroughly vortex to resuspend cells.

Tip: If you used 10 ml of whole blood, wash with 20 ml of Buffer ERL.

5. Centrifuge at 450 x g for 10 min at 4°C. Again, completely remove and discard the supernatant.

6. Add 2 ml TL buffer to the pelleted white blood cells and vortex thoroughly to mix.

7. **Add 150ul of OB protease (D3494) or Protease K at 20mg/ml (D3493) solution, vortex to mix well, and incubate at 55°C in a shaking water bath to effect complete lysis.** If no shaking water bath is available, vortex every 20-30 minutes. Lysis time depend on amount and type of tissue, but usually under 2 hours.

8. Optional: Add 20ul of 25mg/ml RNase A at this point if high level of RNA can be seen from gel analysis.

9. **Add 2.1 ml Buffer BL and vortex to mix. Incubated at 70°C for 10 minutes.** A wispy precipitate may form on addition of Buffer BL, but does not interfere with DNA recovery.

10. **Add 2.2 ml absolute ethanol and mix thoroughly by vortexing.** If precipitation can be seen at this point, break the precipitation by passing through a needle using a syringe.

IMPORTANT: THIS AND ALL SUBSEQUENT STEP MUST BE PERFORMED USING A CENTRIFUGE EQUIPPED WHICH IS CAPABLE OF 6000 X g.

11. Assemble an HiBind® spin column in a 8 ml collection tube (provided). Transfer the 3.5 ml sample from step 10 into the column including any precipitate that may have formed. Centrifuge at 6,000 x g for 10 min to bind DNA. Discard both flow-through

liquid and reuse the collection tube. **Note: Since the column can only contains around 4 ml sample volume, it is necessary to load the column twice.**

12. Place the column back into the 15ml collection tube and load the rest of the sample from step 10 into the column. Centrifuge at 6000 x g for 5 minutes. Discard the flow-through and collection tube.

13. Place the column into a **new 15 ml collection tube** and wash by pipetting 3.5 ml of Buffer HB Buffer. Centrifuge at 6,000 xg for 10 min. **Discard the flow-through liquid and reuse the collection tube**

14. Place the column into same collection tube from step 13 and wash the column by pipetting 3.5 ml of DNA Wash Buffer diluted with ethanol. Centrifuge at 6,000 xg for 5 min. **Discard the flow-through liquid and reuse the collection tube.**

15. Place the column back into same collection tube from step 14 and wash by pipetting 3.5 ml of DNA Wash Buffer diluted with ethanol. Centrifuge at 6,000 x g for 10 min. Reuse collection tube and Discard flow-through liquid.

16. Centrifuge the empty capped column for 10 min at 6000 x g to dry the column matrix. **Do not skip this step - it is critical for removing traces of ethanol that may otherwise interfere with downstream applications. Remove any traces of ethanol from the column using a pipette.**
Option: It might be necessary to dry the column further by placing the column in a vacuum oven at 65C for 10 minutes.

17. Place column into a clean 15 or20 ml centrifuge tube. Add 1-2 ml (depending on desired final concentration) sterile deionized water (or TE buffer) onto the column matrix and centrifuge 5 min at 8000 x g to elute DNA. This represents approximately 70% of bound DNA. An optional second elution will yield any residual DNA, though at a lower concentration. Alternatively, a second elution may be performed using the first eluate to maintain a high DNA concentration. Preheating the water to 70°C and allowing the column to soak 2 min at room temperature before elution may significantly increase yields.

Yield and quality of DNA: determine the absorbance of an appropriate dilution (20- to 50-fold) of the sample at 260 nm and then at 280 nm. The DNA concentration is calculated as follows:

$DNA\ concentration = Absorbance_{260} \times 50 \times (Dilution\ Factor)\ \mu g/ml$
 High copy number plasmids generally yield up to 1 mg of DNA from 500 ml culture. The ratio of $(Abs_{260})/(Abs_{280})$ gives an indication of nucleic acid purity. A value greater than 1.8 indicates greater than 90% nucleic acid. Alternatively, quantity (as well as quality) can sometimes best be determined by agarose gel/ethidium bromide electrophoresis by comparison to DNA samples of known concentrations. Typically, the majority of the DNA eluted is in monomeric supercoil form, though concatamers may also be present.

Troubleshooting Guide

Use the table below to find solutions to any problems you may have with the E.Z.N.A.® Blood RNA Midiprep DNA Kit.

| Problem | Possible Cause | Suggestions |
|-----------------------------|--|--|
| Clogged Column | Incomplete lysis | Extend incubation time of lysis with Buffer TL and protease. Add the correct volume of Buffer BL and incubate for specified time at 70°C. It may be necessary to extend incubation time by 10 min. |
| | Sample too large | If using more than 30 mg tissue, increase volumes of OB Protease or Proteinase K, Buffer TL, Buffer BL, and ethanol. Pass aliquots of lysate through one column successively. |
| | Sample too viscous | Divide sample into multiple tubes, adjust volume to 250 µl with 10 mM Tris-HCl. |
| Low DNA yield | Clogged column | See above |
| | Poor elution | Repeat elution or increase elution volume (see note on page 4). Incubation of column at 70°C for 5 min with Elution Buffer may increase yields. |
| | Improper washing | Wash Buffer Concentrate must be diluted with absolute (100%) ethanol as specified on page 5 before use. |
| Low A_{260}/A_{280} ratio | Extended centrifugation during elution step. | Resin from the column may be present in eluate. Avoid centrifugation at speeds higher than specified. The material can be removed from the eluate by centrifugation — it will not interfere with PCR or restriction digests. |
| | Poor cell lysis due to incomplete mixing with Buffer BL | Repeat the procedure, this time making sure to vortex the sample with Buffer BL immediately and completely. |
| | Incomplete cell lysis or protein degradation due to insufficient incubation. | Increase incubation time with Buffer Tland protease. Ensure that no visible pieces of tissue remain. |
| | Samples are rich in protein. | After applying to column, wash with 300 µl of a 1:1 mixture of Buffer BL and ethanol and then with DNA Wash Buffer. |
| No DNA eluted | Poor cell lysis due to improper mixing with Buffer BL. | Mix thoroughly with Buffer BL prior to loading HiBind® column. |

| Problem | Possible Cause | Suggestions |
|--|---|---|
| | Poor cell and/or protein lysis in Buffer TL. | Tissue sample must be cut or minced into small pieces. Increase incubation time at 65°C with Buffer TL to ensure that tissue is completely lysed. |
| | Absolute ethanol not added to Buffer BL. | Before applying sample to column, an aliquot of Buffer BL/ethanol must be added. See protocol above. |
| | No ethanol added to Wash Buffer Concentrate. | Dilute Wash Buffer with the indicated volume of absolute ethanol before use. |
| Washing leaves colored residue in column | Incomplete lysis due to improper mixing with Buffer BL. | Buffer BL is viscous and the sample must be vortexed thoroughly. |
| | No ethanol added to Wash Buffer Concentrate. | Dilute Wash Buffer with the indicated volume of absolute ethanol before use. |

If the above suggestions fail to resolve any problems you are having with the E.Z.N.A.® Blood DNA Kit, please feel free to fax our technical specialists at:

US customers: 800-832-8896 or 770-931-8400

All other customers: (770) 931-0230

Or direct your questions via E-mail to info@omegabiotek.com.