NEBNext® Ultra™ DNA Library Prep Protocol for Illumina® With Size Selection (E7370) Version 2

Isabel Gautreau

Abstract

This protocol is a group of the following 5 methods from the NEB website:
1. NEBNext End Prep
2. Adaptor Ligation
3. Size Selection of Adaptor-ligated DNA
4. PCR Amplification
5. Cleanup of PCR Amplification

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Guidelines

The NEBNext Ultra DNA Library Prep Kit for Illumina contains enzymes and buffers that are ideal to convert a small amount of DNA input into indexed libraries for next-generation sequencing on the Illumina platform (Illumina, Inc).

Adaptor Ligation:

If DNA input is < 100 ng, dilute the NEBNext Adaptor for Illumina* 1:10 and use immediately to a final concentration of 1.5 µM.

Size Selection:

This protocol is suitable for input amounts of 50ng or higher. For lower input amounts we recommend that the protocol without size selection be followed.

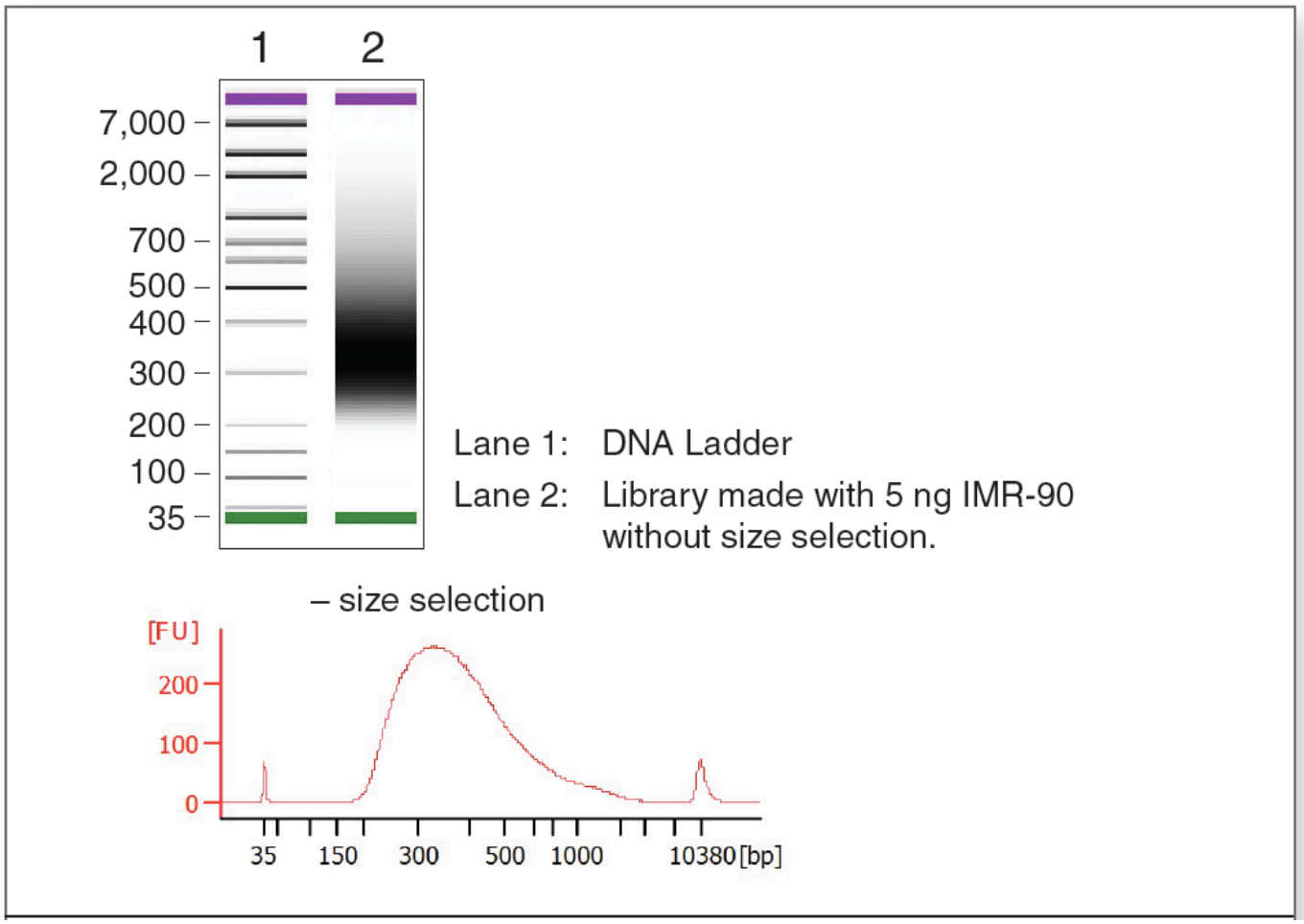
This size selection is for libraries with 200 bp inserts only and is based on a starting volume of 100 µl. For libraries with different size fragment inserts, refer to Table 1 for the appropriate volume of beads to be added.

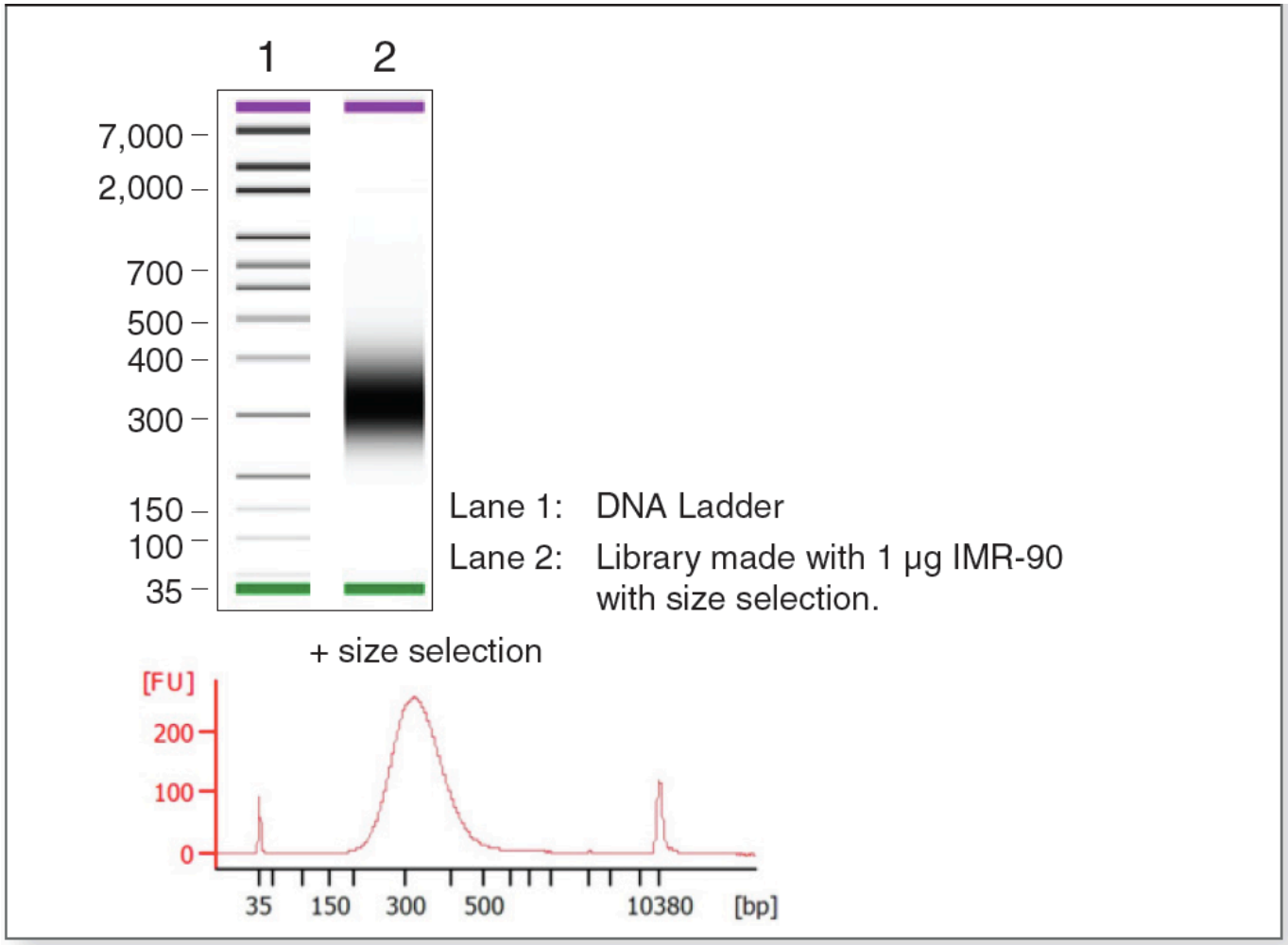
Table 1: Recommended conditions for bead based size selection.

LIBRARY PARAMETERS	APPROXIMATE INSERT SIZE	150 bp	200 bp	250 bp	300-400 bp	400-500 bp	500-700 bp
	Total Library Size (insert + adaptor)	270 bp	320 bp	400 bp	400-500 bp	500-600 bp	600-800 bp

VOLUME TO BE ADDED (µl)	1st Bead Selection	65	55	45	40	35	30
	2nd Bead Selection	25	25	25	20	15	15

Figure 1: Examples of libraries prepared with human IMR-90 gDNA.





Materials

NEBNext Ultra DNA Library Prep Kit for Illumina - 24 rxns E7370S by New England Biolabs

Protocol

NEBNext End Prep

Step 1.

Mix components

Mix the following components in a sterile nuclease-free tube (Total volume **65 µl)**:

Reagent	Cap Color	Volume
Fragmented DNA		55.5 µl
End Prep Enzyme Mix	Green	3.0 µl

End Repair Reaction Buffer (10x)	Green	6.5 µl

REAGENTS

End Prep Enzyme Mix E7371 in Kit E7370 or E7442 by New England Biolabs

End Repair Reaction Buffer E7372 in Kit E7370 or E7442 by New England Biolabs

NOTES

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Starting Material: 5 ng–1 µg fragmented DNA.

Isabel Gautreau 31 Mar 2017

Set a 100 or 200 µl pipette to 50 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect the liquid from the side of the tube. Note: it is important to mix well. The presence of a small amount of bubbles will not interfere with the performance. See the guidelines section for a picture of acceptable amounts of bubbles.

NEBNext End Prep

Step 2.

Place in a thermocycler, with the heated lid set to ≥ 75°C, and run the following program:

Time	Temperature
30 minutes	20°C
30 minutes	65°C
Hold	4°C

Adaptor Ligation

Step 3.

Add the following components directly to the End Prep reaction mixture:

Reagent	Cap Color	Volume
Blunt/TA Ligase Master Mix	Red	15 µl
Ligation Enhancer	Red	1 µl
NEBNext Adaptor For Illumina	Red	2.5 µl

REAGENTS

Blunt/TA Ligase Master Mix E7373 in Kit E7370 or E7445 by New England Biolabs

Ligation Enhancer E7374 in Kits E7370 or E7445 by New England Biolabs

NEBNext Adaptor for Illumina E7337 in Kits E7335, E7500, E771 by New England Biolabs

NOTES

Isabel Gautreau 12 Jan 2017

The NEBNext adaptor is provided in NEBNext Singleplex (NEB #E7350) or Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E6609, #E7600) Oligos for Illumina.

if DNA input is < 100 ng, dilute the NEBNext adaptor for Illumina (provided at 15 µM) 10 fold in 10 mM Tris HCl with 10 mM NaCl to a final concentration of 1.5 mM,. Use immediately.

Ligation enhancer and Blunt TA Ligase Master Mix can be mixed ahead of time are is stable for at least 8 hours at 4°C. We do not recommend adding adaptor to a premix in the adaptor ligation step. For best results add adaptor last and immediately mix well, or premix adaptor and sample and then add the other ligation reagents.

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Set a 100 or 200 µl pipette to 50 µl and then pipette the entire volume up and down at least 10 times to mix thoroughly. Perform a quick spin to collect the liquid from the side of the tube. Note: it is important to mix well. The presence of a small amount of bubbles will not interfere with the performance. See the guidelines section for a picture of acceptable amounts of bubbles.

Adaptor Ligation

Step 4.

Incubate at 20°C for 15 minutes in a thermal cycler.

DURATION

00:15:00

Adaptor Ligation

Step 5.

Add 3 µl of USER™ enzyme to the ligation mixture.

AMOUNT

3 µl Additional info:

REAGENTS

NEBNext USER In Index Kits by New England Biolabs

NOTES

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USER addition and USER incubation are only required for use with NEBNext adaptors.

USER is provided in the NEBNext Index Primer kits. It is also available as a separate product NEB# M5505 which can be used in NEBNext library prep workflows.

Adaptor Ligation

Step 6.

Mix well and incubate at 37°C for 15 minutes with heated lid set to ≥ 47°C.

DURATION
00:15:00

NOTES
Isabel Gautreau 14 Mar 2017

Samples can be stored overnight at -20°C, for longer term storage the samples should be cleaned up or size selected.

If moving on to PCR in the same day, take the NEBNext Q5 Hot Start HiFi PCR Master Mix at room temperature during size selection or cleanup. Once thawed, gently mix by inverting the tube several times.

Size Selection

Step 7.

Vortex SPRIselect beads to resuspend. AMPure XP beads can be used as well. If using AMPure XP beads, please allow the beads to warm to room temperature for at least 30 minutes before use.

NOTES
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Please note: *the volumes of SPRIselect or AMPure XP reagent provided here are for use with the sample contained in the exact buffer at this step. These volumes may not work properly for a size selection at a different step in the workflow, or if this is a second size selection at this step. For size selection of samples contained in different buffer conditions the volumes may need to be experimentally determined.*

! ***The following size selection protocol is for libraries with 200 bp inserts only. For libraries with different size fragment inserts, refer to the Table1 in the Guidelines for the appropriate volumes of beads to be added. The size selection protocol is based on a starting volume of 100 µl. Size selection conditions were optimized with AMPure XP beads; however, SPRIselect beads can be used following the same conditions.***

Insert/ total library size; first/ second bead addition volume

150/ 270; 65/25

200/ 320; 55/25

250/ 400; 45/25

300-400/ 400-500; 40/20

400-500/ 500-600; 35/15

500-700/ 600-800; 30/15

Size Selection

Step 8.

Add 13.5 µl dH2O to the ligation reaction for a 100 µl total volume.

Size Selection

Step 9.

For 200 bp inserts add 55 µl (0.55 x) of resuspended SPRIselect beads to the 100 µl ligation reaction. Mix well by pipetting up and down at least 10 times.

NOTES

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Be careful to expel all of the liquid out of the tip during the last mix. Vortexing for 3-5 seconds on high can also be used. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

Size Selection

Step 10.

Incubate samples on bench top for at least 5 minutes at room temperature.

DURATION

00:05:00

Size Selection

Step 11.

Place the tube/plate on an appropriate magnetic stand to separate the beads from the supernatant. If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.

DURATION

00:05:00

NOTES

Isabel Gautreau 23 Jan 2017

If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.

Size Selection

Step 12.

After 5 minutes (or when the solution is clear), carefully transfer the supernatant containing your DNA to a new tube **(Caution: do not discard the supernatant)**. Discard the beads that contain the unwanted large fragments.

Size Selection

Step 13.

For 200 bp libraries, add 25 µl (0.25 x) resuspended SPRIselect beads to the supernatant and mix *at least 10 times.* Then incubate samples on the bench top for at least 5 minutes at room temperature.

DURATION
00:05:00

NOTES
Isabel Gautreau 14 Mar 2017

Be careful to expel all of the liquid from the tip during the last mix.

Size Selection

Step 14.

Place the tube/plate on an appropriate magnetic stand for 5 minutes to separate the beads from the supernatant.

DURATION
00:05:00

NOTES
Isabel Gautreau 12 Jan 2017

If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.

Size Selection

Step 15.

After 5 minutes (or when the solution is clear), carefully remove and discard the supernatant that contains unwanted DNA. **(Caution: do not discard beads).**

NOTES
Isabel Gautreau 23 Jan 2017

Be careful not to disturb the beads that contain the desired DNA targets

Size Selection

Step 16.

Add 200 µl of 80% freshly prepared ethanol to the tube/plate while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

DURATION
00:00:30

NOTES
Isabel Gautreau 14 Mar 2017

Be careful not to disturb the beads that contain DNA targets.

Size Selection
Step 17.

Repeat the previous step once. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of ethanol with a p10 pipette tip.

DURATION
00:00:30

Size Selection
Step 18.

Air the dry beads for **up to** 5 minutes while the tube is on the magnetic stand with the lid open. **Caution: Do not overdry the beads.**

NOTES
Isabel Gautreau 23 Jan 2017

Caution: Do not overdry the beads. This may result in lower recovery of DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

Size Selection
Step 19.

Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads into 17 µl of 10 mM Tris-HCl or 0.1X TE.

Size Selection
Step 20.

Mix well on a vortex mixer or by pipetting up and down 10 times. Incubate for at least 2 minutes at room temperature.

DURATION
00:02:00

NOTES
Isabel Gautreau 23 Jan 2017

If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand.

Size Selection
Step 21.

Place the tube/plate on a magnetic stand. After 5 minutes (or when the solution is clear), transfer 15 µl to a new PCR tube for (amplification).

DURATION
00:05:00

NOTES
Isabel Gautreau 12 Jan 2017

Try to avoid any bead carryover.

Size Selection
Step 22.

Place the tube/plate on a magnetic stand. After 5 minutes (or when the solution is clear), transfer 15 µl to a new PCR tube for (amplification).

NOTES
Lenny Teytelman 10 Nov 2015
According to the comments, this is a safe stopping point. After the ligated fragments have been purified, it's ok to freeze overnight and start the PCR amplification step the following day.
Isabel Gautreau 21 Mar 2017

SAFE STOP: Samples can be stored at -20ºC

PCR Amplification
Step 23.

Mix the following components in sterile strip tubes (**Total volume 50 µl**):

For index kits with primers separate:

Reagent	Cap Color	Volume
Adaptor Ligated Fragments	n/a	15 µl
NEBNext Q5 Hot Start HiFi PCR Master Mix	Blue	25 µl
Index Primer/ I7 primer	Blue	5 µl
Universal PCR primer/ I5 Primer	Blue	5 µl

For Index kits where the primers are already combined:

Reagent	Cap Color	Volume
Adaptor Ligated Fragments	n/a	15 µl
NEBNext Q5 Hot Start HiFi PCR Master Mix	Blue	25 µl
Index/ Universal Primer		10 µl

REAGENTS

NEBNext® Q5® Hot Start HiFi PCR Master Mix E6625 or M0543 by New England Biolabs

NEBNext Index Primers E7335 or E7500 or E7710 or E7730 by New England Biolabs

NOTES

Isabel Gautreau 12 Jan 2017

The primers are provided in NEBNext Singleplex (NEB #E7350) or Multiplex (NEB #E7335, #E7500, #E7710, #E7730, #E6609, #E7600) Oligos for Illumina.

Check that your primers are 10 µM (tube label).

PCR Amplification

Step 24.

PCR using the following cycling conditions:

Cycle Step	Temperature	Time	Cycles
Initial Denaturation	98°C	30 Seconds	1
Denaturation	98°C	10 Seconds	4-12*
Annealing/ Extension	65°C	75 Seconds	
Final Extension	65oC	5 Minutes	1
Hold	4°C	∞	

* Please note the number of PCR cycles recommended in this table are to be seen as s starting point to determine the number of PCR cycles best for your samples. The number of cycles should be chosen based on the input amount and sample type. Thus, samples prepared with a different method prior to library prep may require further re-optimization of the number of PCR cycles. The number of cycles should be high enough to provide sufficient library fragments for a successful sequencing run, but low enough to avoid PCR artifacts and over-cycling (high molecular weight fragments on Bioanalyzer).

DNA Input	Number of PCR Cycles
1 µg	4

50 ng	7-8
5 ng	12

The NEBNext adaptors contain a unique truncated design. Libraries constructed with NEBNext adaptors require a minimum of 3 amplification cycles to add the complete adaptor sequences.

Cleanup of PCR Amplification

Step 25.

Vortex AMPure XP or SPRIselect beads to resuspend.

NOTES

Isabel Gautreau 05 Apr 2017

If using AMPure XP beads, allow the beads to warm to room temperature for at least 30 minutes before use.

Cleanup of PCR Amplification

Step 26.

Add 45 µl (0.9X) resuspended SPRIselect beads to the PCR reaction. Mix well by pipetting up and down at least 10 times.

NOTES

Isabel Gautreau 05 Apr 2017

Be careful to expel all of the liquid out of the tip during the last mix. Vortexing for 3-5 seconds on high can also be used. If centrifuging samples after mixing, be sure to stop the centrifugation before the beads start to settle out.

Cleanup of PCR Amplification

Step 27.

Incubate for at least 5 minutes at room temperature.

DURATION

00:05:00

Cleanup of PCR Amplification

Step 28.

Place the tube/plate on an appropriate magnetic stand to separate the beads from the supernatant (5 min or when the solution is clear).

DURATION

00:05:00

NOTES
Isabel Gautreau 05 Apr 2017

If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing on the magnetic stand.

Cleanup of PCR Amplification
Step 29.

After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets (**Caution do not discard beads**).

Cleanup of PCR Amplification
Step 30.

Wash #1: Add 200 µl of 80% freshly prepared ethanol to the tube/ plate while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

DURATION
00:00:30

NOTES
Isabel Gautreau 05 Apr 2017

Be careful not to disturb the beads that contain DNA targets.

Cleanup of PCR Amplification
Step 31.

Wash #2: Repeat the previous once for a total of two washes. Be sure to remove all visible liquid after the second wash. If necessary, briefly spin the tube/plate, place back on the magnet and remove traces of ethanol with a p10 pipette tip.

DURATION
00:00:30

Cleanup of PCR Amplification
Step 32.

Air dry the beads for up to 5 minutes while the tube/plate is on the magnetic stand with the lid open.

Caution: Do not over-dry the beads. This may result in lower recovery of DNA target. Elute the samples when the beads are still dark brown and glossy looking, but when all visible liquid has evaporated. When the beads turn lighter brown and start to crack they are too dry.

Cleanup of PCR Amplification
Step 33.

Remove the tube/plate from the magnetic stand. Elute the DNA target from the beads by adding 33 µl of 0.1X TE.

Cleanup of PCR Amplification

Step 34.

Mix well by pipetting up and down 10 times, or on a vortex mixer. Incubate for at least 2 minutes at room temperature.

DURATION
00:02:00

NOTES
Isabel Gautreau 05 Apr 2017

If necessary, quickly spin the sample to collect the liquid from the sides of the tube or plate wells before placing back on the magnetic stand.

Cleanup of PCR Amplification

Step 35.

Place the tube/plate on the magnetic stand. After 5 minutes (or when the solution is clear), transfer 28 µl to a new PCR tube for and store at -20oC.15

DURATION
00:05:00

Cleanup of PCR Amplification

Step 36.

Check the size distribution on an Agilent Bioanalyzer® High Sensitivity DNA chip. The sample may need to be diluted before loading.

Warnings

This protocol is suitable for input amounts of 50 ng or higher. For lower input amounts we recommend that the protocol without size selection be followed.