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XCeloSeq® Targeted cfDNA Enrichment V2
Laboratory Protocol and Record

Please ensure that you have completely read the full protocol before starting, **IFU2115 XCeloSeq Targeted cfDNA Enrichment V2 – Protocol**.

Operating Procedure

10.1. ATO Reaction V2 – Step 1: ATO and cfDNA Mixture

Dilute your samples (if required) and combine them with the components as listed in the table below:

Order	Component	Tube cap colour	Volume per reaction (µl)	Added
1	cfDNA Sample	--	Up to 17.0	☐
2	Molecular Biology Grade Water	--	Variable	☐
3	ATO	Blue	1.5	☐
4	ATO Reaction Buffer	Amber	1.5	☐
	Total		20.0	

Seal the PCR vessel, mix by vortexing, and centrifuge briefly. Every well should be sealed tightly to avoid evaporation and sample loss. Incubate the mixture in a thermocycler as detailed in the table below, with a heated lid ≥100 °C

ATO Reaction – Step 1: Incubation Conditions

Stage	Temperature (°C)	Duration (min)
1	98	2
2	10	1
3	10	Hold

10.2. ATO Reaction V2 – Step 2: Addition of ATO Reaction Enzyme

Remove the sample from the thermocycler, briefly spin down and carefully open the PCR vessel. Add components to product from previous step as detailed below:

Order	Component	Tube cap colour	Volume per single reaction (µl)	Added
1	Product from **Step 10.1**	N/A	20.0	--
2	ATO Reaction Enzyme	Green	2.0	☐
	Total		22.0	

Seal the PCR vessel, mix by vortexing, and centrifuge briefly. Ensure that the thermocycler is precooled to 4 °C. Place your PCR vessel into the machine and continue to thermocycle as detailed in the table at the top of the following page, with a heated lid ≥100 °C

ATO Reaction V2 – Step 2: Incubation Conditions

Step	Cycles	Temperature (°C)	Duration (min)
1	1	4	Hold/Pause
2		10	1
3		26	12
4		30	20
5	2	65	1
6		10	1
7		26	12
8		30	20
9	1	4	Hold

10.3. Amplification One

Remove the samples from the thermocycler, briefly spin down and carefully open the PCR vessel. Add components to product from previous step as detailed below:

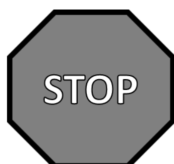
Order	Component	Tube cap colour	Volume per single reaction (µl)	Added
1	Product from **Step 10.2**	--	22.0	--
2	Amplification One Mix	Transparent	26.0	☐
3	Primers	Red	2.0	☐
	Total		50.0	

Vortex the final Amplification One mixture and centrifuge briefly. Incubate the mixture in a thermocycler as detailed in the table below, with a heated lid ≥100 ^{o}C

Amplification One: Incubation Conditions

Stage	Cycles	Temperature (°C)	Duration
1	1	37	10 min
2	1	98	30 s
3	15	98	5 s
4		60	30 s
5		72	30 s
6	1	72	2 min
7	Hold	4	Hold

Safe Stopping Point



After the incubation step has completed samples can be stored at -20°C overnight. Samples must proceed to Amplification Two within 24 hours.

If stored at –20 °C, ensure samples are at room temperature, vortexed and centrifuged before proceeding

10.4. Amplification Two: Target-Specific PCR

Take the previous product and prepare two 22 µl aliquots in fresh PCR vessels.

Prepare the Pool-1 Outer Amplification Two Reaction Mixture by adding reagents to the **first aliquot**, according to the order in the table below:

Order	Component	Tube cap colour	Volume per single reaction (µl)	Added
1	**Aliquot 1** of **Step 10.2** Product	--	22.0	--
2	Pool 1 – Outer	Orange	3.0	☐
3	Master Mix	Lilac	25.0	☐
	Total		50.0	

Prepare the Pool-2 Outer Amplification Two Reaction Mixture by adding reagents to the **second aliquot**, according to the order in the table below:

Order	Component	Tube cap colour	Volume per single reaction (µl)	Added
1	**Aliquot 2** of **Step 10.2** Product	--	22.0	--
2	Pool 2 – Outer	White	3.0	☐
3	Master Mix	Lilac	25.0	☐
	Total		50.0	

Vortex the Amplification Two Mixtures and centrifuge briefly. Incubate the mixture in a thermocycler as detailed in the table below, with a heated lid ≥100 ^{o}C

Amplification Two: Incubation Conditions

Stage	Cycles	Temperature (°C)	Duration
1	1	98	30 s
2		98	5 s
3	14	65	5 min
4		72	30 s
5	1	72	2 min
6	Hold	4	Hold

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10.5. Bead Purification

Before starting it is necessary to ensure that fresh 80% ethanol is prepared ready for use. Also ensure XCeloSeq Purification Beads have already come to room temperature and have been completely resuspended by vortexing prior to use

Bind Amplification Two products to Purification Beads

1)	Add **90 µl** of **Purification Beads** to each reaction. Vortex well or mix by pipetting each sample 15 times to generate a homogenous mixture of beads and sample that is consistent in colour	☐
2)	Leave samples at room temperature for **5 minutes**. Once the incubation is complete, spin down briefly to collect sample in the bottom of the vial.	☐
3)	Place the samples on a magnet for **3 minutes** or until all the beads have been collected.	☐
4)	Without disturbing the collected beads, use a pipette to collect and dispose of the supernatant. If the beads are disturbed, dispense the supernatant back into the vessel and repeat the incubation to allow them to collect on the side of the tube again.	☐

Wash beads twice with 80% ethanol

	Wash the samples by repeating steps 5 – 7 two times:	Wash 1	Wash 2
5)	While leaving the vial on the magnet, add **150 µl** of freshly prepared **80% ethanol.**	A ☐	A ☐
6)	Incubate for **30 seconds.** Ensure all beads remain gathered to the side of the vial.	B ☐	B ☐
7)	Carefully discard the supernatant without disturbing the beads.	C ☐	C ☐

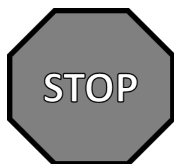
Dry beads and remove residual ethanol

8)	After carefully discarding the second supernatant, leave the vials for **2 minutes** at room temperature to allow residual ethanol to collect at the bottom of the vessel.	☐
9)	Carefully inspect each well and without disturbing the beads completely remove all traces of the ethanol. **Note:** Residual ethanol is a PCR inhibitor and must not be carried over into subsequent reactions. A well-dried bead pellet appears dry but retains a rich, solid brown colour.	☐
10)	Allow the beads to air dry for **3 minutes**. If surfaces of the well do not appear dry, remove any droplets of ethanol with a fresh pipette tip and then incubate for a further **2 minutes**.	☐

Elute samples from beads

11)	To elute samples, remove from the magnet and add **23 µl** of either **molecular biology grade H_2O** or **10 mM Tris-HCL pH 8.0**. Thoroughly resuspend the beads in the eluent by vortexing or pipetting to form a homogenous suspension. Incubate the fully resuspended beads for **5 minutes** at room temperature. **Note:** Dry beads should resuspend quickly and easily. Over-dry beads appear cracked, dry, and flaky and may require a longer time to resuspend by extending the time spent vortexing or being pipetted.	☐
12)	Place the samples on the magnet for **3 minutes**, or until all the beads have been collected.	☐
13)	Carefully transfer **21 µl** of the eluted amplification product into a clean PCR tube	☐

Safe Stopping Point



After the bead purification samples can be stored at –20 °C overnight.

Samples must proceed to Amplification Three within 24 hours.

If stored at –20 °C, ensure samples are at room temperature, vortexed and centrifuged before proceeding.

10.6. Amplification Three: Nested Target-Specific PCR

Prepare the Pool - 1 Inner Amplification Three Reaction Mixture by adding reagents to the bead-purified sample from **Step 10.4**, according to the order in the table below:

Order	Component	Tube cap colour	Volume per single reaction (µl)	Added
1	**Pool 1 – Outer** product of **Step 10.4**	--	21.0	--
2	Pool 1 – Inner	Black	2.0	☐
3	Master Mix	Lilac	25.0	☐
4	Pre-mixed UDI Primers	White	2.0	☐
	Total		48.0	

Prepare the Pool - 2 Inner Amplification Three Reaction Mixture by adding reagents to the bead-purified sample from **Step 10.4**, according to the order in the table below:

Order	Component	Tube cap colour	Volume per single reaction (µl)	Added
1	**Pool 2 – Outer** product of **Step 10.4**	--	21.0	--
2	Pool 2 – Inner	Yellow	2.0	☐
3	Master Mix	Lilac	25.0	☐
4	Pre-mixed UDI Primers	White	2.0	☐
		Total	50.0	

Vortex the Amplification Three Reaction Mixtures and centrifuge briefly

Incubate the mixture in a thermocycler as detailed in the table below, with a heated lid ≥100 °C:

Amplification Three: Incubation Conditions

Stage	Cycles	Temperature (°C)	Duration
1	1	98	30 s
2	Variable, See Next Table	98	5 s
3		65	5 min
4		72	30 s
5	1	72	2 min
6	Hold	4	Hold

Suggested initial cycle numbers are shown in the table below.

Amplification Three: Recommended Amplification Conditions

Input DNA Quantity	Recommended Amplification Three cycle number for cfDNA
5 ng	16
10 ng	15
20 ng	14
50 ng	13

10.7. Bead Purification

Before starting it is necessary to ensure that fresh 80% Ethanol is prepared ready for use. Also ensure XCeloSeq Purification Beads have already come to room temperature and have been completely resuspended by vortexing prior to use

Bind Amplification Three products to Purification Beads

1)	Add **60 µl** of **Purification Beads** to each reaction. Vortex well or mix by pipetting each sample 15 times to generate a homogenous mixture of beads and sample that is consistent in colour	☐
2)	Leave samples at room temperature for **5 minutes**. Once the incubation is complete, spin down briefly to collect sample in the bottom of the vial.	☐
3)	Place the samples on a magnet for **3 minutes** or until all the beads have been collected.	☐
4)	Without disturbing the collected beads, use a pipette to collect and dispose of the supernatant. If the beads are disturbed, dispense the supernatant back into the vessel and repeat the incubation to allow them to collect on the side of the tube again.	☐

Wash beads twice with 80% ethanol

	Wash the samples repeating steps 5 – 7 two times:	Wash 1	Wash 2
5)	While leaving the vial on the magnet add **150 µl** of freshly prepared **80% ethanol.**	A ☐	A ☐
6)	Incubate for **30 seconds.** Ensure all beads remain gathered to the side of the vial.	B ☐	B ☐
7)	Carefully discard the supernatant without disturbing the beads.	C ☐	C ☐

Dry beads and remove residual ethanol

8)	After carefully discarding the second supernatant leave vials for **2 minutes** at room temperature to allow residual ethanol to collect at the bottom of the vessel.	☐
9)	Carefully inspect each well and without disturbing the beads completely remove all traces of the ethanol. **Note:** Residual ethanol is a PCR inhibitor and must not be carried over into subsequent reactions. A well-dried bead pellet appears dry but retains a rich, solid brown colour.	☐
10)	Allow the beads to air dry for **3 minutes**. If surfaces of the well do not appear dry, remove any droplets of ethanol with a fresh pipette tip and then incubate for a further **2 minutes**.	☐

Elute samples from beads

11)	To elute samples, remove from the magnet and add **32 µl** of **TE** (10 mM Tris-HCL pH 8.0 containing 0.1 to 1mM EDTA). Thoroughly resuspend the beads in the eluent by vortexing or pipetting to form a homogenous suspension. Incubate the fully resuspended beads for **5 minutes** at room temperature. **Note:** Dry beads should resuspend quickly and easily. Over-dry beads appear cracked, dry, and flaky and may require a longer time to resuspend by extending the time spent vortexing or being pipetted.	☐
12)	Place the samples on the magnet for **3 minutes**, or until all the beads have been collected.	☐
13)	Carefully transfer **30 µl** of the eluted amplification product into a clean PCR tube	☐

This table is used as a record for which UDI Pair was added to each sample.

Sample Number	Sample Name	UDI Pair Name
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Sample Number	Sample Name	UDI Pair Name
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