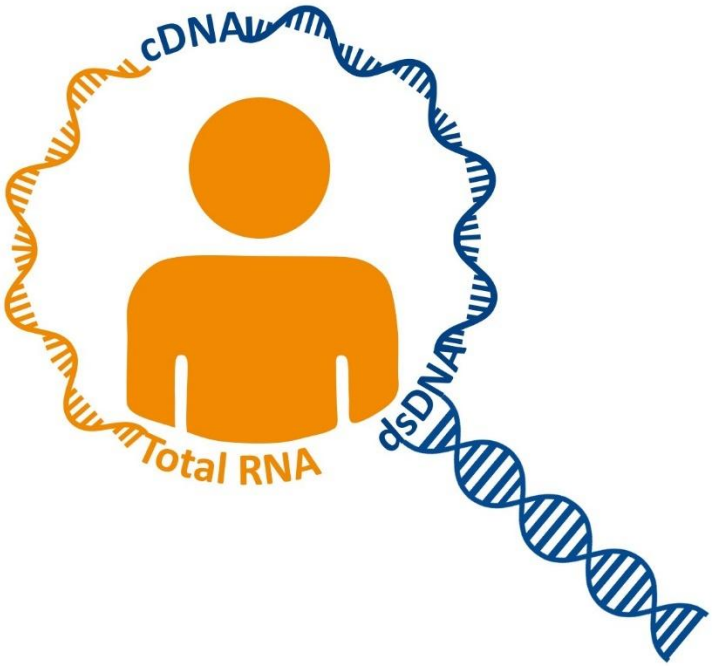
User Manual

ClickTech Library Kit full-length mRNA_Seq V2.0



Version 3.0

Ordering information

(for detailed kit content see **Table 1**)

Kit Name: ClickTech Library Kit full-length mRNA_Seq V2.0

Product Number	Product Name	Application
BCK-LFRS-V2.0	ClickTech Library Kit full-length mRNA_Seq V2.0	Production of full-length mRNA derived cDNA libraries for sequencing applications.

For References, FAQs and ordering please see online or contact us:

online: www.baseclick.eu
orders: orders@baseclick.eu
support: support@baseclick.eu
phone: +49 89 9699 3401

ClickTech Library Kit full-length mRNA_Seq V2.0

Introduction and product description:

baseclick's ClickTech Library Kit full-length mRNA_Seq V2.0 has been developed as a method for sequencing total mRNA or the whole exome of cells for genetic diagnosis. The kit provides reagents for cDNA synthesis from any RNA pool and introduces a single azide nucleotide at the 3'End of cDNA. This azido-modified cDNA can react with an 5'-alkyne-modified adapter sequence in a highly specific and biorthogonal manner under benign click reaction conditions. With the included specially designed PCR Primers, the 3'-adapter-clicked cDNA can be amplified *via* PCR, enabling subsequent long-read Third Generation Sequencing experiments or alternatively targeted gene amplifications

For preparation of full-length mRNA libraries

The ClickTech Library Kit full-length mRNA_Seq V2.0 contains reagents to perform 10 full-length mRNA library preparations.

For research use only.

Information in this document is subject to change without notice. baseclick GmbH assumes no responsibility for any errors that may appear in this document.

baseclick GmbH disclaims all warranties with respect to this document, expressed or implied, including but not limited to those of merchantability or fitness for a particular purpose. In no event shall baseclick GmbH be liable, whether in contract, tort, warranty, or under any statute or on any other basis for special, incidental, indirect, punitive, multiple or consequential damages in connection with or arising from this document, including but not limited to the use thereof.

Please read the material safety data sheets (MSDS) provided for each product/component available online at www.baseclick.eu.

The product is protected by proprietary licence.

Literature Citation: When describing a procedure for publication using this product, please refer to it as the *ClickTech Library Kit full-length mRNA_Seq V2.0.*

1. Materials provided with the Kit and storage conditions

Table 1: Contents of the Kit and storage conditions.

Color code	Quantity	Component	Storage	Thaw Before Use
colorless	15 µL	dNTP Mix (10 mM)	–20 °C	2 - 8 °C
blue	15 µL	LFRS Primer RT (100 µM)	–20 °C	2 - 8 °C
yellow	35 µL	10x RNase H Buffer	–20 °C	2 - 8 °C
yellow	16 µL	RNase H (5 U/µL)	–20 °C	n.r.
yellow	15 µL	RNase A (10 mg/mL)	–20 °C	n.r.
red	60 µL	5x Azide Elongation Buffer	–20 °C	2 - 8 °C
red	15 µL	3'-N_3-ddGTP (10 mM)	–20 °C	2 - 8 °C
red	22 µL	Azide Elongase (20 U/µL)	–20 °C	n.r.
green	30 µL	5x Activator[2]	–20 - +20°C	n.r.
green	11	Reactor XS	–20 - +20°C	n.r.
green	10 µL	Alkyne Adapter (100 µM)	–20 °C	2 - 8 °C
purple	130 µL	2x baseclick PCR Master Mix	–20 °C	2 - 8 °C
purple	15 µL	LFRS Primer Forward (10 µM)	–20 °C	2 - 8 °C
purple	15 µL	LFRS Primer Reverse (10 µM)	–20 °C	2 - 8 °C

2. Required Material and Equipment not included in this kit

- RNA pool of interest
- RNase free water (e.g. DEPC-treated water)
- RNase free microcentrifuge tubes and pipette tips
- RNase away or similar to clean surfaces
- Reverse Transcriptase, we recommend the use of Superscript IV Reverse Transcriptase
- Thermal cycler
- Thermal mixer
- Polyacrylamide or agarose gel electrophoresis
- Appropriate spin column-based nucleic acid purification system such as MinElute PCR Purification Kit from Qiagen

3. Workflow

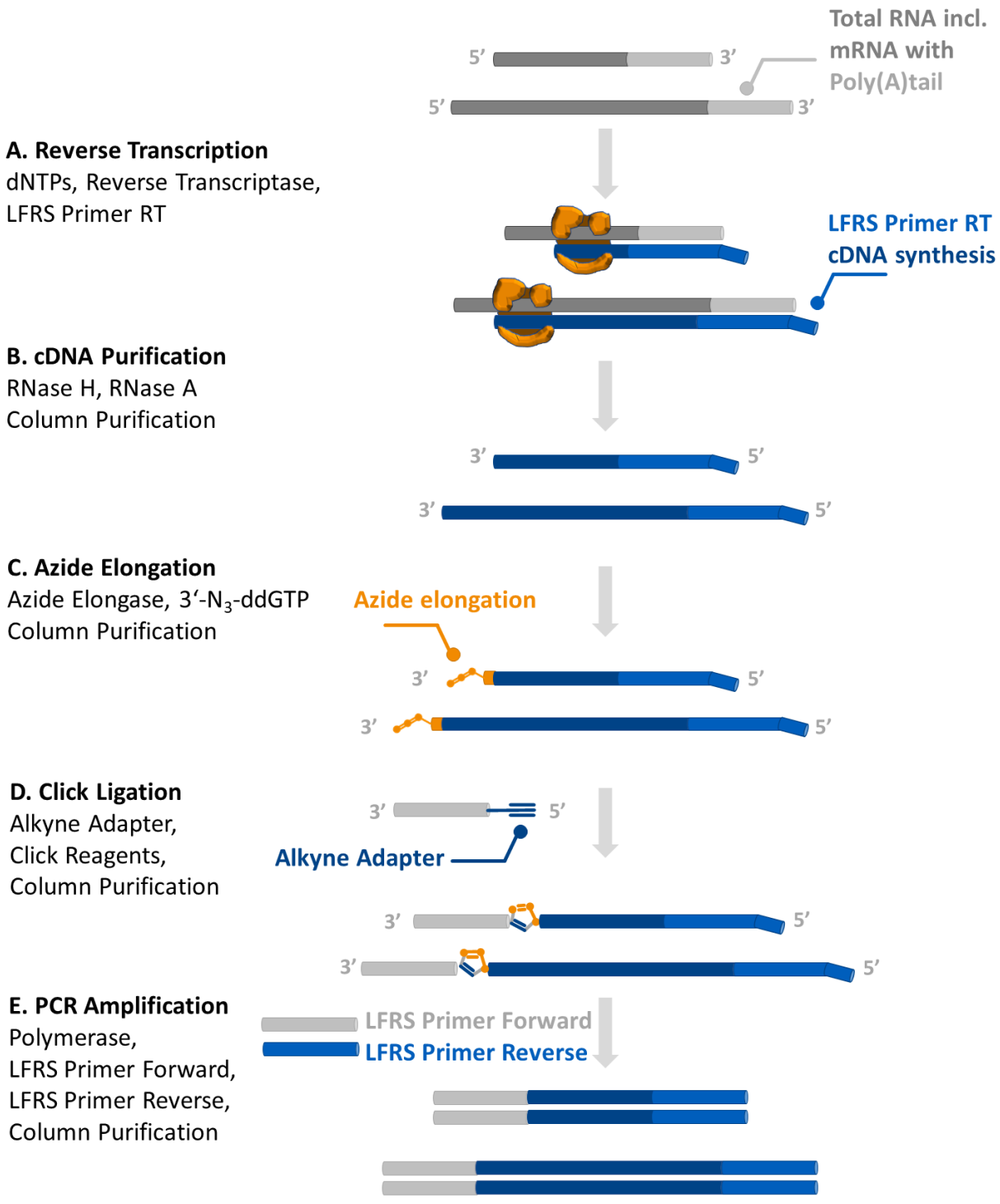


Figure 1: General workflow of the ClickTech Library Kit full-length mRNA_Seq V2.0.

4. Reverse Transcription and cDNA Preparation

In general, 1 µg of total RNA pool or 0.6-1 µg singular mRNA is suitable in the following protocol. To prevent contamination of RNase, always wear gloves and use dedicated solutions e.g. RNase AWAY to clean pipettes and maintain a clean working area. For a proper workflow please take care that all specified solutions are thawed before usage.

4.1 Hybridization of Poly(dT) LFRS Primer RT

- Thaw the dNTP Mix on ice and spin down briefly.
- Thaw the LFRS Primer RT on ice and spin down briefly.
- Prepare the reaction mixture at room temperature:

Table 2: Reagents and quantities for Hybridization.

Color code	Quantity	Component
Not included	1 µg or 0.6-1 µg	Total RNA Pool mRNA
colorless	1 µL	dNTP Mix (10 mM)
blue	1 µL	LFRS Primer RT (100 µM)
Not included	to 13 µL total volume	RNase-free H_2O

- Mix by pipetting, spin down briefly.
- For hybridization heat the mixture for 5 min at 65 °C and cool down for 3 min at 0°C.
- Promptly proceed with step **4.2**, Reverse Transcription.

4.2 Reverse Transcription

The following protocol is created using Invitrogen`s SuperScript™ IV Reverse Transcriptase. Please adapt the process if needed for the use of different reverse transcriptases.

- Keep the Reverse Transcriptase in an enzyme cooler (–20 °C) or on ice.
- Thaw the Reverse Transcription Buffer (5x) on ice and spin down briefly.
- Thaw the DTT (100 mM) on ice and spin down briefly.
- Add the following volumes to the reaction prepared before in step **4.1**.

Table 3: Reagents and quantities for Reverse Transcription.

Color code	Quantity	Component
see step 4.1	13 µL	Reaction mixture from step 4.1
Not included	4 µL	5x Reverse Transcription Buffer
Not included	1 µL	DTT (100 mM)
Not included	1 µL	Reverse Transcriptase (200 U/µL)
Not included	1 µL	RNase-free H_2O or Recombinant RNase Inhibitor (e.g. RNaseOUT™ from Invitrogen™)
-	total volume 20 µL	x

- Mix by pipetting, spin down briefly.
- For cDNA synthesis heat the mixture for 20 min at 50 °C, 10 min at 80 °C and cool down for 3 min at 4 °C.

5. cDNA Purification

After cDNA synthesis, RNA digestion and spin-column purification are necessary to remove RNA, excess dNTPs, and all enzymes.

5.1 RNA Digestion

- Thaw the 10x RNase H Buffer on ice and spin down briefly.
- Keep the RNase H in an enzyme cooler (–20 °C) or on ice.
- Keep the RNase A in an enzyme cooler (–20 °C) or on ice.
- Prepare the reaction mixture at room temperature:

Table 4: Reagents and quantities for RNA Digestion.

Color code	Quantity	Component
see step 4.2	20 µL	Reaction mixture from step **4.2**
yellow	3 µL	10x RNase H Buffer
yellow	1 µL	RNase A (10 mg/mL)
yellow	1.4 µL	RNase H (5 U/µL)
Not included	4.6 µL	Nuclease-free H_2O
-	total volume 30 µL	x

- Mix by pipetting and spin down briefly.
- Heat the mixture for 30 min at 37 °C, 15 min at 65 °C and cool down for 3 min at 4 °C. Proceed with step **5.2** Spin Column Purification.

5.2 Spin Column Purification

After RNA digestion, the mixture obtained needs to be purified using an appropriate spin column-based nucleic acid purification system (**not included**). To obtain a final volume of 17 µL use ca. 19 µL of Elution Buffer. If a minimum elution volume of 17 µL is not possible, then the volume of 5x Azide Elongation Buffer used in the subsequent step should be adjusted accordingly to obtain a 1x concentrated Buffer solution.

IMPORTANT: Please check if the Elution Buffer provided in your chosen spin column-based nucleic acid purification system contains EDTA. If so, pure H_2O should be used for the elution step, rather than Elution BufferAt this point the purified cDNA can be maintained at –20 °C for long-term storage.

6. Azide Elongation

6.1 Azide Elongation

- Thaw the Azide Elongation Buffer on ice and spin down briefly.
- Thaw the 3'-N_3-ddGTP on ice and spin down briefly.
- Keep the Azide Elongase in an enzyme cooler (–20 °C) or on ice.
- Prepare the following reaction mixture at room temperature:

Table 5: Reagents and quantities for Azide Elongation.

Color code	Quantity	Component
see step 5.2	17 µL	Reaction mixture from step **5.2**
red	5 µL	5x Azide Elongation Buffer
red	1 µL	3'-N_3-ddGTP (10 mM)
red	2 µL	Azide Elongase (20 U/µL)
Not included	Total volume 25 µL	If necessary, fill up with H_2O

- Mix by pipetting, spin down briefly.
- For Azide Elongation heat the mixture for 90 min at 37 °C and cool down for 3 min at 4 °C.
- Proceed with step **6.2** Column Purification.

6.2 Spin Column Purification

After Azide Elongation, the mixture obtained needs to be purified using an appropriate spin column-based nucleic acid purification system (**not included**). To obtain a final volume of 9 µL use ca. 11 µL of Elution Buffer. If a minimum elution volume of 9 µL is not possible, then the volume of 5x Activator² used in the subsequent step should be adjusted accordingly to obtain the same overall concentration as specified below.

IMPORTANT: Please check if the Elution Buffer provided in your chosen spin column-based nucleic acid purification system contains EDTA. If so, pure H_2O should be used for the elution step, rather than Elution Buffer.

At this point the purified cDNA can be maintained at –20 °C for long-term storage.

7. Click Ligation

7.1 Click Ligation

- Place the 5x Activator² at room temperature and mix by vortexing. This process can take up to 30 minutes for the first time. If precipitate is still present, please continue vortexing until completely dissolved. Afterwards we recommend storing the 5x Activator² at room temperature to avoid precipitation.
- Thaw the Alkyne Adapter on ice and spin down briefly.
- Prepare the reaction mixture at room temperature:

Table 6: Reagents and quantities for Click Ligation.

Color code	Quantity	Component
see step 6.2	9 µL	Reaction mixture from step **6.2**
green	2 pellets	Reactor XS
green	2.5 µL	5x Activator²
green	0.5 µL	Alkyne Adapter (100 µM)
Not included	Fill up to 12.5 µL	H_2O

- Mix by pipetting and spin down briefly to ensure that the pellets are immersed in the reaction mixture.
- To initiate the click reaction, close the vial and incubate the mixture in a thermomixer at 45 °C, 600 rpm for 1.5 h.
- After the reaction, transfer the supernatant to a new vial (without pellets!). Wash the pellets with an additional 12.5 µL water (mix by pipetting) and combine this supernatant with the supernatant from the reaction.
- Proceed with step **7.2** Spin Column Purification.

7.2 Spin Column Purification

After Click Ligation, the mixture obtained needs to be purified using an appropriate spin column-based nucleic acid purification system (**not included**), to obtain a final volume of 10-30 µL using Elution Buffer.

At this point the purified cDNA can be maintained at –20 °C for long-term storage.

8. PCR Amplification

At this point, proceeding with the instructions below will allow users to amplify a given cDNA pool using **untargeted Primers** (LFRS Primer Forward and LFRS Primer Reverse) to obtain a **full length** mRNA library.

Notice: If desired, **sequence specific amplifications** are also possible, and can be achieved by replacing LFRS Primer Forward and LFRS Primer Reverse with independently designed DNA primers (**not included**). To this end, the consensus coding sequence (CCDS) of your desired mRNA sequence should be used as a reference.

8.1 Full-Length PCR Amplification

In a 200 µL tube, add all reaction components as listed in **Table 7**.

Table 7: Reagents and quantities for PCR to obtain full length amplificons.

Color code	Quantity	Finale Concentration	Component
see step 7.2	3-9 ng	x	Purified, clicked-cDNA step **7.2**
purple	1 µL	0.4 µM	LFRS Primer Forward (10 µM)
purple	1 µL	0.4 µM	LFRS Primer Reverse (10 µM)
purple	12.5 µL	1x	2x baseclick PCR Master Mix
Not included	Fill up to 25 µL	x	Nuclease-free H_2O

- Mix by pipetting, spin down briefly.
- Transfer the mixture to the thermal cycler block with the lid temperature set to 105 °C and begin thermocycling using the program shown in **Table 8**.

Table 8: Thermocycling conditions for PCR Amplification.

Step	Temperature	Time
Initial Denaturation	94 °C	30 seconds
35 Cycles	94 °C 57 °C 65 °C	20 seconds 30 seconds 1-2 minutes*
Final Extension	65 °C	10 minutes
Hold	4 °C	∞

* By increasing or decreasing the extension time, PCR fragments are shifted towards longer or shorter amplicons (please calculate with 1kb/min).

8.2 Spin Column Purification

The mixture obtained should once more be purified using an appropriate spin column-based nucleic acid purification system (**not included**).

At this point the purified cDNA can be maintained at –20 °C for long-term storage.

9. Exemplary Results

These are exemplary results from using the components and protocols of the kit for exome sequencing.

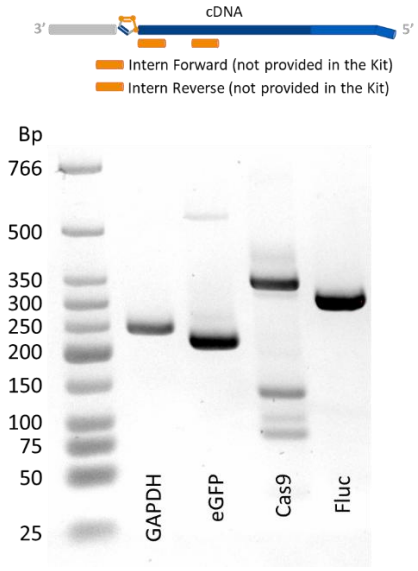


Figure 2: Agarose gel electrophoresis showing PCR fragments that were generated from a click-ligated cDNA pool using this kit. The "InternalForward" Primers (not included) cover the very 5'-end of the transcript and the second primer (Intern Reverse) binds to a gene specific reverse complement. In addition to house-keeping gene GAPDH, mRNAs coding for eGFP, Cas9 and Fluc were spiked into a Jurkat cell total mRNA pool for internal control. All PCR products have the desired fragment size.

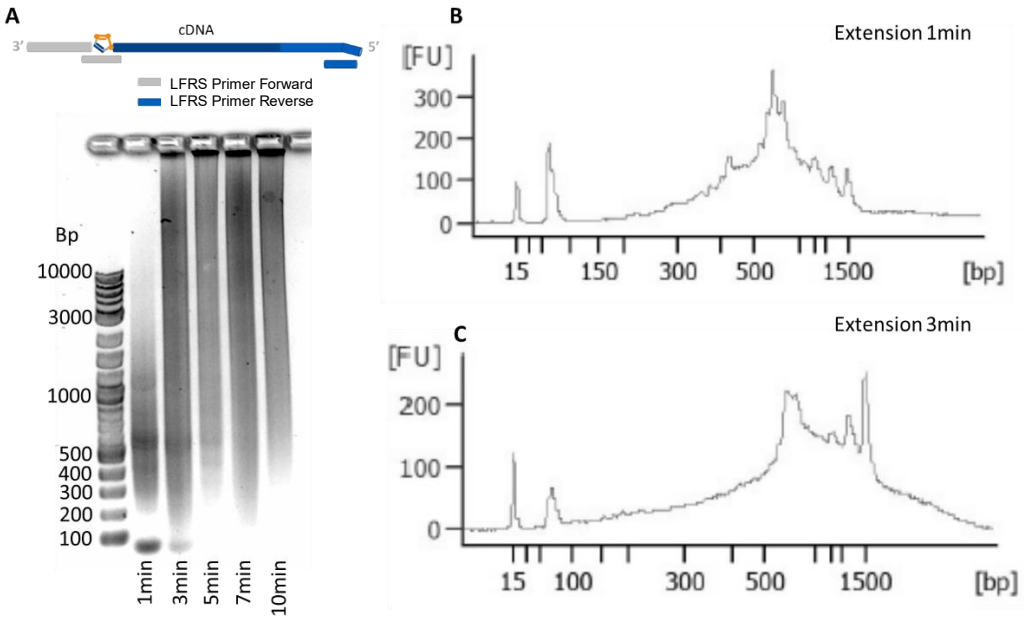


Figure 3: Agarose gel electrophoresis (A) and Bioanalyzer (B, C) measurements obtained from full length PCR fragments that were generated from using click-ligated cDNA Jurkat cell pool cDNA using this kit. One primer binds to the click ligated adapter (LFRS Primer Forward), while the second primer (LFRS Primer Reverse) matches the LFRS Primer RT used for priming of the reverse transcription. Cycling conditions as described in Table 8 differ in extension time 1 min, 3 min, 5 min, 7 min and 10 min. Depending on the extension time, the distribution of the PCR fragments shift towards longer amplicons.

Trouble Shooting

Table 9: Trouble Shooting solutions.

Problem	Possible Reason	Solution
Low cDNA synthesis yield	Contamination of RNase during cDNA synthesis.	Wear gloves and use dedicated solutions e.g. RNase AWAY to clean pipettes and tips and maintain a clean working area. Ensure that all reagents are RNase free.
Poor performance of the click reaction	Poor Azide Elongation yield because of secondary structures.	Preheat the cDNA mixture up to 65 °C for 10 min and cool it down immediately to 4 °C. Add the Azide Elongation mixture and go ahead.
	Poor Azide Elongation yield due to higher concentrated starting material.	Check if the yield can be increased by increasing the amount of Azide Elongase, 3'-N_3-ddGTP or reaction time.
	After Click ligation the supernatant was not transferred before washing the copper pellets.	Washing step must be carried out after transferring the supernatant and must be carried out with water.
	Column Purification was carried out using the Elution Buffer provided in a given PCR Purification Kit.	Some standard commercial buffer components can decrease the click reaction efficiency or even impair reaction process. For example, TE buffer contains ethylenediaminetetraacetic acid (EDTA), which can chelate Cu(II) ions and decrease the reaction rate. Make sure not to use TE buffer for elution of the purified cDNA before click labeling. Thiol groups from reducing agents like β-mercaptoethanol or dithiothreitol (DTT) can stop the click reaction.
Column purification: Low or no recovery of cDNA	Too much cDNA product applied to the spin column.	Check the manufacturer's specifications to ensure the maximum amount of material is not being exceeded.
	Incomplete elution of cDNA.	Make sure that the elution solution has been completely absorbed by the silica column membrane before centrifugation.
	The size of cDNA too large for the given spin column.	Check the manufacturer's specifications to ensure the maximum length of the cDNA is not being exceeded.
Poor performance in the downstream applications	Salt residue remains in eluted cDNA.	Wash the silica column twice with Wash Buffer.
PCR amplicons don't have the desired size	Extension time of the PCR cycling is too short	For full length amplification increase the extension time (Calculation 1 kb/1 min). See also **Figure 3**.